RAPPORT FINAL

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Analyse spatiale et fonctionnelle de la diversité d'un système symbiotique en milieux insulaire et continental : cas du *Pterocarpus officinalis* Jacq. et de ses microorganismes associés en forêt marécageuse

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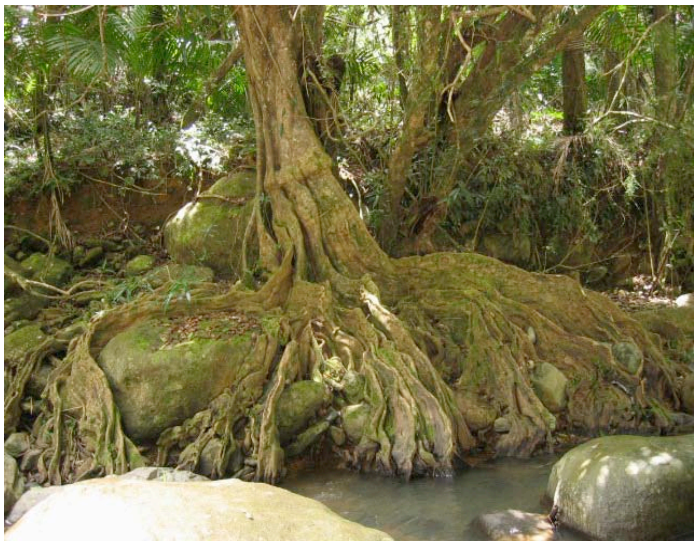

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1. Résumé

La structuration de la diversité génétique et le fonctionnement des organismes impliqués dans les symbioses (rhizobiums et champignons mycorhiziens à arbuscules) de *Pterocarpus officinalis* Jacq. sont étudiés selon différentes échelles spatio-temporelles et en fonction de contraintes édaphiques (inondation et salinité) dans des forêts marécageuses de la région Caraïbes. La structuration de la diversité des populations de cet arbre est analysée sur 202 individus provenant de huit populations insulaires (1 population en Dominique, 1 population en Martinique, 2 populations à Porto Rico et 4 populations en Guadeloupe) et d'une population de Guyane française. Nous avons utilisé huit marqueurs microsatellites nucléaires pour leur polymorphisme (4 à 20 allèles) chez *P. officinalis,* et identifié 3 locus microsatellites chloroplastiques à partir d'amorces universelles. Les paramètres de diversité chloroplastique varient nettement entre les populations. Ainsi, la population de Moule en Guadeloupe présente les plus faibles variations (H_{cp}=0,22), et celle d'Indian River en Dominique les plus fortes variations (H_{cp}=0,68). Dans tous les cas les populations insulaires montrent des valeurs inférieures à celles de la population de Guyane. Ce résultat est conforté par l'analyse des microsatellites nucléaires. Les indices de structuration observés avec les marqueurs chloroplastique et nucléaire, respectivement F_{stcp}= 0,58 et F_{stnuc}= 0,29, sont liés à un faible taux de dissémination du pollen et des graines (Rp/s= 2.18). On pourrait donc penser que les graines jouent un rôle prépondérant dans la dissémination de cette espèce.

Toutes les souches bactériennes isolées de nodules des différentes populations de *P. officinalis* appartiennent au genre *Bradyrhizobium*. L'analyse phylogénétique des *Bradyrhizobium* par séquençage de l'ITS montre d'une part, un groupe monophylétique contenant la grande majorité des souches insulaires très proches d'une souche ouest-africaine de référence et, d'autre part, deux groupes paraphylétiques comprenant les souches de Guyane française, mais aussi certaines souches de Porto Rico. La diversité bactérienne au niveau insulaire est plus faible que celle de Guyane française.

L'absence de spores dans des sols marécageux ne permet pas d'identifier les champignons mycorhiziens à arbuscules (CMA) inféodés à *P. officinalis*. Dans ces sols, les CMA sont présents sous forme de mycélium libre et/ou en symbiose avec *P. officinalis*. Le potentiel mycorhizien des sols marécageux et le taux de colonisation des racines de *P. officinalis* diminuent le long du gradient de salinité en saison sèche (2-26‰) comme en saison humide (5-22‰). Les CMA de *P. officinalis* seraient tolérants jusqu'à 10‰ de sel.

Des études expérimentales montrent que l'inoculation avec *Bradyrhizobium* a un effet bénéfique sur la production de biomasse du *Pterocarpus* cultivé en l'absence de NaCl. À 10‰ de NaCl, la biomasse des plants inoculés diminue à cause de la baisse du nombre et de l'activité fixatrice d'azote des nodules. De plus, les ions Cl^- et Na^+ se sont accumulés davantage dans les racines que dans les feuilles, atténuant ainsi leur effet toxique sur la plante. Par contre, une forte mortalité des plants est observée à 20‰ de NaCl.

Les plants *P. officinalis* ont remarquablement bien poussé en milieu inondé grâce à la formation de racines adventives, d'aérenchymes et de lenticelles sur la partie submergée des tiges. L'inondation induit également la formation de nodules de tige et de mycorhizes à

arbuscules sur les racines adventives. La souche de CMA, *G. intraradices*, se révèle efficace sur la biomasse totale et l'acquisition de P dans les feuilles de *P. officinalis* grâce à une colonisation mycorhizienne tout à fait remarquable en milieu inondé. Cependant, l'acquisition de P n'améliore pas la fixation d'azote des nodules de tige et racine des *Pterocarpus*.

2. Abstract

We propose to determine the genetic diversity, structure and function of *Pterocarpus officinalis* Jacq. and their microsymbionts (rhizobia and arbuscular mycorrhizal fungi) at the spatial and temporal levels, and following soil conditions (flooding and salinity) in swamp forests within Caribbean basin. The genetic diversity and structure were analysed on leaves from 202 individuals of 9 *P. officinalis* populations dispersed on different islands (1 population from Dominica, 1 population from Martinique, 2 populations from Puerto Rico and 4 populations from Guadeloupe) in the Caribbean and in one population of French Guyana in the South American continent. We used six nuclear microsatellite loci (displaying a number of alleles ranging from 4 to 20) designed specifically for *P. officinalis* and three universal chloroplast probes to genotype individuals. For chloroplast microsatellite marker, the diversity parameters varied markedly among the populations. The population of Le Moule (Guadeloupe) showed the lowest values (H_{cp}= 0.22) and the population of Indian River (Dominica) the highest values (H_{cp}= 0.63). In all cases, island populations showed a lower value than French Guyana population. These results are in accordance with data from nuclear microsatellite markers. The fixation index for both chloroplast and nuclear microsatellites ranged from F_{stcp}= 0.58 to F_{stnuc}= 0.29, respectively, resulting in a low pollen seed mediated ratio (Rp/s= 2.18). Therefore, seed could play a major role in the *P. officinalis* dispersal.

All the strains isolated from nodules of the different populations of *P. officinalis* belong to the phylogenetic group of *Bradyrhizobium*. Phylogenetic analysis of the ITS rDNA gene sequences from *Bradyrhizobium* strains showed also three groups, one monophyletic group including all the island strains together with the *Bradyrhizobium* sp. V from West Africa, and two paraphyletic groups including all the French Guyana strains and some Puerto Rico strains. However, island *Bradyrhizobium* strains showed a lower diversity than those from French Guyana.

We did not identify arbuscular mycorrhizal (AM) fungi of *P. officinalis*, because no spores were found in soil cores collected within swamp forests. We suggest that propagules were only mycelium pieces and/or root fragments of colonized *Pterocarpus* roots. The mycorrhizal soil infectivity (MPN values) and AM colonization of *P. officinalis* decrease along the salinity gradient where the salt ranged from 26 to 2‰ and from 22 to 5‰ in dry and wet seasons, respectively. The AM fungi may be adapted until the salt levels of 8 to 10‰ were reached in wet and dry seasons, respectively.

The inoculation of *P. officinalis* seedlings by two *Bradyrhizobium* strains (UAG OM2 and UAG OM6) improved biomass production of plants in the absence of salt. At 10‰ of NaCl, the decrease of biomass production of seedlings could be due to the reduction of the number and nitrogen fixation of nodules. In thus, the Na+ and Cl- contents were higher in roots than in leaves of seedlings. This could attenuate the toxic effects of Na+ and Cl- in seedlings. At 20‰ of NaCl, the ion contents were similar in roots and leaves, and provoked a severe mortality of seedlings.

Nodule formation both on adventitious roots and stem as well as AM colonization of adventitious roots were well developed on *P. officinalis* seedlings under flooding. The AM fungus, *Glomus intraradices*, contributed noteworthy to the flood-tolerance of seedlings by improving their growth and P acquisition in leaves. Nevertheless, P acquisition did not improve nitrogen-fixing stem and root nodules of *P. officinalis* seedlings.

3. Rappel du contexte scientifique et des objectifs du projet

Dans la région Caraïbe (îles et continent), la forêt marécageuse à *Pterocarpus officinalis* est une formation remarquable qui se développe sur des sols inondés ou temporairement inondés en arrière de la mangrove, en bordure de rivière et dans des dépressions humides en montagne (Eusse & Aide, 1999 ; Imbert *et al*., 2000). Dans les Antilles, l'espèce peut représenter jusqu'à 90 % de la strate arborée (Alvarez-Lopez, 1990). En Guadeloupe, la forêt marécageuse à *P. officinalis* couvre environ 2600 ha dont 460 ha dans la réserve naturelle du Grand-cul-de-sac Marin où elle fait l'objet d'un statut de protection (Imbert *et al*., 2000). Cette forêt marécageuse est en régression à cause des effets conjugués de l'extension des infrastructures routières et des activités agricoles. *P. officinalis* a été exploité en Guadeloupe pour alimenter les distilleries jusqu'au milieu du siècle. Actuellement, cet arbre est exploité par les Saramacas de Guyane qui l'ont adopté pour ses qualités remarquables dans l'artisanat.

Les forêts marécageuses à *P. officinalis* sont peu connues aussi bien en ce qui concerne leur structuration que leur dynamique en particulier dans le milieu insulaire (Rivera-Ocasio *et al*., 2002). Un des objectifs de ce projet était d'analyser la structuration de la diversité infra-spécifique de *P. officinalis* à différentes échelles spatio-temporelles et en fonction des contraintes du milieu. Le milieu insulaire présente à cet égard des caractéristiques géographiques et biologiques très spécifiques (syndrome d'insularité). Il permet également de comprendre les effets de la fragmentation sur la structuration de la diversité génétique des espèces forestières.

La diversité des symbioses fixatrices d'azote et mycorhiziennes est un aspect très peu étudié sur *P. officinalis* (Saur *et al*., 1998 ; Bâ *et al*., 2004). Aucune étude n'avait porté sur la diversité génétique conjointe des partenaires de ces deux types de symbiose. Un autre objectif de ce projet était d'évaluer parallèlement la diversité infra-spécifique des populations de *P. officinalis* et des microorganismes associés en forêt marécageuse dans différents sites en milieu insulaire (Guadeloupe, Marie-Galante, Martinique, Dominique et Porto Rico) et continental (Guyane française), et en fonction de contraintes édaphiques (salinité et inondation).

En période d'inondation, les sols marécageux sont hypoxiques et parfois anoxiques. Ces conditions sont défavorables à une bonne dégradation de la matière organique et donc à la minéralisation de l'azote et du phosphore organique. La dénitrification et le lessivage accentuent aussi les carences en azote et en phosphore (Barrios & Herrera, 1993). En période d'exondation, l'évapotranspiration entraîne des remontées de sel par capillarité. Un troisième objectif de ce projet était d'étudier le rôle des rhizobiums et des CMA sur l'adaptation du *P. officinalis* à l'inondation et à la salinité.

4. Résultats

Pour étudier la diversité génétique et la structuration de *P. officinalis* dans la région Caraïbe, nous avons analysé 202 individus provenant de 8 populations insulaires et d'une population de Guyane française (Muller *et al*., 2006 a). Nous avons développé spécifiquement et sélectionné huit marqueurs microsatellites nucléaires pour leur polymorphisme (4 à 20 allèles) chez *P. officinalis.* Ce sont des marqueurs codominants, faciles à mettre en œuvre, et particulièrement efficaces quand une faible diversité génétique est attendue. En parallèle, 3 locus microsatellites chloroplastiques ont été identifiés à partir d'amorces universelles. Les résultats obtenus à partir des deux types de marqueurs microsatellites sont récapitulés dans le tableau 2 (Muller *et al*., 2006 b, en annexe). Les paramètres de diversité chloroplastique varient nettement entre les populations. Ainsi, la population de Moule en Guadeloupe est celle qui présente les plus faibles variations (H_{cp}=0,22), alors que la plus variable est celle d'Indian River en Dominique (H_{cp}=0,68). Dans tous les cas, comme on pouvait s'y attendre pour des populations présentes sur des îles de petite taille, les populations insulaires montrent des valeurs inférieures par rapport aux populations continentales. Ce résultat est conforté par les microsatellites nucléaires qui montrent, eux aussi une diversité plus importante sur le continent que dans les îles. Les index de fixation, varie de F_{is} =-0,043 à F_{is} =0,368, pour les populations de Deshaies et du Galion, respectivement. Un déficit en hétérozygote significatif a été observé dans toutes les populations étudiées, ce qui peut être interprété comme le résultat d'un effet fondation dans les petites îles ou dans les populations isolées. Les indices de structuration observés avec les marqueurs chloroplastique et nucléaire, respectivement F_{stcp}= 0,58 et F_{stnuc}= 0,29, sont importants et à relier à un faible taux de dissémination du pollen et des graines. Cependant, les flux de graine par rapport au flux de pollen (Ennos, 1994), indiquent une valeur de P/G= 2,88 pour toutes les populations confondues, ce qui est très inférieur aux valeurs retrouvées généralement chez des populations continentales d'Angiospermes chez qui le pollen est le moyen de dissémination génétique préférentiel. On peut donc penser que les graines jouent un rôle prépondérant dans la dissémination de cette espèce, résultat qui a été confirmé par la viabilité décroissante de graines de *P. officinalis* ayant séjourné jusqu'à un mois dans l'eau de mer (Muller *et al.*, 2006 b).

Parallélement, nous avons isolé et caractérisé les bactéries de nodules des différentes populations de *P. officinalis* (Muller *et al.*, 2006 c). Toutes les souches isolées appartiennent au genre *Bradyrhizobium* (Figure 1). L'analyse phylogénétique des *Bradyrhizobium* par séquençage de l'ITS laisse cependant apparaître la présence de trois clades distincts. Nous observons un groupe monophylétique contenant la grande majorité des souches insulaires (Guadeloupe, Martinique et Dominique, et une bonne majorité des souches Porto Ricaine) très proches d'une souche ouest-africaine de référence, provenant de zone aride. Les deux autres groupes sont quant à eux, paraphylétiques et contiennent les souches continentales (Guyane française), mais aussi certaines souches Porto Ricaine (Figure 1). Toujours est-il que la diversité bactérienne au niveau insulaire est plus faible que celle observée en milieu continental (Guyane). Trois hypothèses peuvent être formulées au vu de ces résultats.

Premièrement, on peut s'interroger sur la pertinence des marqueurs ITS en ce qui concerne la détection de diversité en milieu insulaire, sans pour autant remettre en cause la variabilité présente en milieu continental. Il a été montré récemment qu'une très forte divergence génomique globale peut exister sans pour autant qu'une divergence des séquences ITS soit détectée. C'est le cas de deux souches de *Bradyrhizobium* photosynthétiques intégralement séquencées qui, malgré une différence d'environ 11% de la taille de leur génome, montrent 100% d'homologie au niveau de leurs séquences ITS (Moulin pers. com. 2006). Ainsi, la faible divergence entre les souches insulaires n'est pas forcement corrélée à

une faible divergence génomique globale. D'autant que les premiers résultats de champs pulsés suggèrent qu'il existerait de la diversité chez les souches insulaires pourtant identiques lors du séquençage de leurs ITS. Dans tous les cas, il existe un différentiel entre continent et îles, puisque les souches des îles présentes pour certaines 100% d'homologie avec la souche génomique V de *Bradyrhizobium*, alors qu'aucune souche des îles n'est commune à la Guyane.

Deuxièmement, la faible diversité observée en milieu insulaire pourrait être fruit d'un effet sélectif majeur de la part du couvert végétal. En effet, *P. officinalis* représente près de 90% de la strate arborée des populations insulaires, et près de l'intégralité des légumineuses nodulées. La population continentale de Guyane est caractérisée par une plus faible densité en *P.officinalis*, mais présente un grand nombre de légumineuses en terme de genre, *P. officinalis* constituant environ 25% de la strate arborée, et 37,5% des légumineuses nodulées. Cette différence de composition floristique pourrait influencer directement les communautés microbiennes et leur distribution comme tendent à le monter certaines expériences (Zak *et al*., 2003) mais aussi favoriser grandement un type bactérien en terme de valeur sélective de par la relation intime exercée dans le cadre de la symbiose légumineuses/bactéries. Ce phénomène pourrait être exacerbé par les contraintes édaphiques rencontrées en milieu insulaire. Il nous faut prendre aussi en compte les effets démographiques directement liés aux aspects insulaires tels que les effets de fondations, de migrations inter-îles, de taille efficace des populations insulaires pouvant influencer la dérive génétique.

Le troisième point sur lequel on peut s'interroger reste la forte homologie entre les souches insulaires et des souches originaires d'Afrique de l'Ouest. Ce dernier point est peut être moins aberrant qu'il n'y paraît si l'on se réfère aux résultats récents obtenus sur la diversité des microorganismes contenus dans le milieu aérien (Maron *et al*., 2005), et les particularités du transport aérien de microorganisme entre l'Afrique de l'Ouest et la Caraïbe (Prospero *et al*., 2005), permettant d'observer des fortes similitudes génétiques entre les microorganismes ouest-africains et caribéens (Griffing *et al.*, 2001 ; Griffing *et al.*, 2003 ; Kellogg *et al*., 2004 ; Prospero *et al*., 2005).

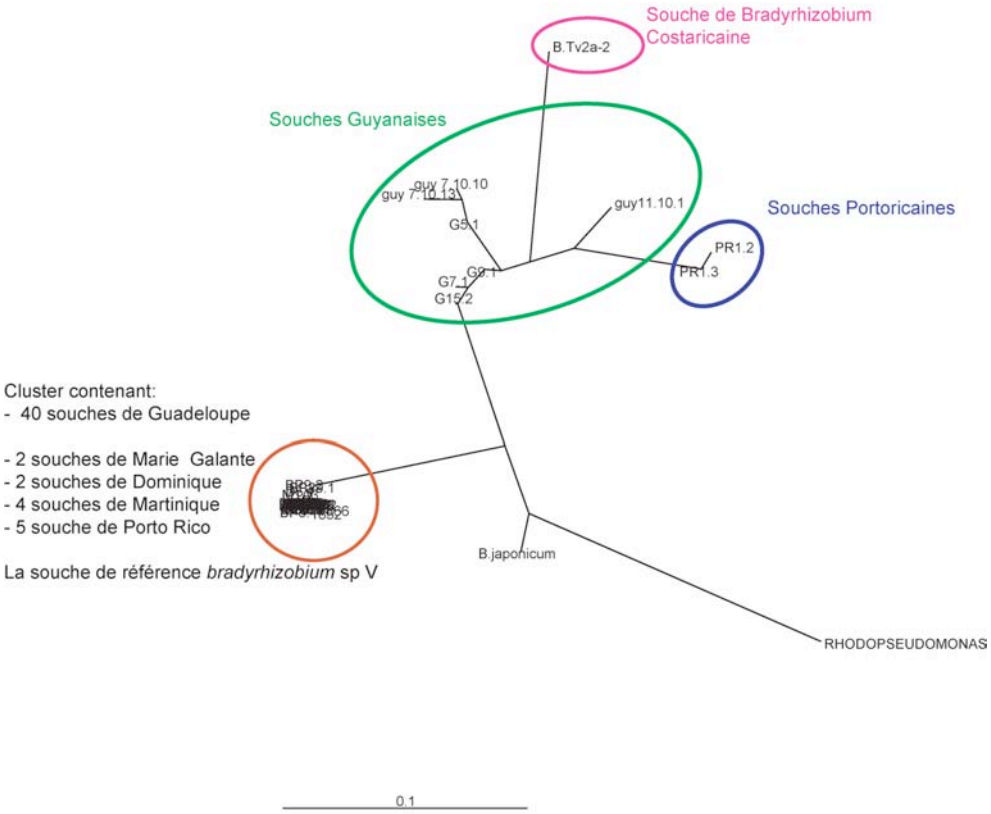

Figure 1. Arbre phylogénétique non enraciné des séquences ITS de différentes souches de *Bradyrhizobium* provenant d'îles, du continent, et de séquences ITS de souches de référence (*B.* sp V, *B.* tv2a-2, *B. japonicum*, *Rhodopseudomonas*) (Muller *et al*., 2006 c).

Outre les *Bradyrhizobium*, *P. officinalis* est colonisé par des champignons mycorhiziens à arbuscules (CMA) (Bâ *et al*., 2004 ; Saint-Etienne *et al*., 2006). L'absence de spores dans les sols marécageux n'a pas permis l'identification taxonomique des champignons mycorhiziens à arbuscules inféodés à *P. officinalis*. Dans la forêt de Belle Plaine (Guadeloupe), les CMA sont présents uniquement sous forme de mycélium libre et/ou en symbiose avec *P. officinalis* dans les sols marécageux. Le potentiel mycorhizien du sol et le taux de colonisation des racines de *P. officinalis* ont diminué le long d'un gradient de salinité en saison sèche (2-26‰) et en saison humide (5-22‰). Cependant, les CMA de *P. officinalis* seraient tolérants jusqu'à 10‰ de sel.

Nous avons étudié la tolérance au NaCl (0, 10 et 20‰) de *P. officinalis* en symbiose avec deux souches de *Bradyrhizobium* provenant de milieu salé ou non (Dulormne *et al*., en préparation). En l'absence de sel, l'inoculation a eu un effet bénéfique sur la production de biomasse du *Pterocarpus*. À 10‰ de NaCl, la biomasse des plants inoculés est comparable à celle des plants non inoculés à cause de la baisse du nombre de nodules et de l'activité fixatrice d'azote. Cette dose de NaCl n'a d'ailleurs pas affecté la production de biomasse des plants non inoculés. Les ions Cl^- et Na^+ se sont accumulés davantage dans les racines que dans les tiges et les feuilles du *P. officinalis*. L'effet toxique des ions Na^+ et Cl^- dans les

feuilles est atténué par leur rétention dans les racines sans qu'on sache clairement les mécanismes de régulation. Ces résultats sont en accord avec nos observations de terrain qui montrent une prolifération de plantules sous le houppier de l'arbre à des concentrations de sels proches de 10‰ et, par contre, une faible régénération dans les zones plus salées de l'arrière mangrove (forte mortalité des plantules de *P. officinalis* à 20‰).

P. officinalis est également soumis à l'inondation pendant une partie de l'année. Nous avons étudié le rôle de *Bradyrhizobium* sp. et de *Glomus intraradices* sur la tolérance à l'inondation de *P. officinalis* en serre (Fougnies *et al*., 2006). *P. officinalis* a poussé mieux en milieu inondé qu'en milieu exondé indépendamment de l'inoculation. L'apparition de racines adventives, d'aérenchymes et de lenticelles a été tout à fait remarquable sur la partie submergée des tiges. Les plantules de *P. officinalis* ont donc développé des mécanismes d'adaptation à l'inondation comparables à ceux des plantes aquatiques (Liao & Lin, 2001). Chez les plants inoculés, l'inondation a induit aussi la formation de nodules caulinaires et de mycorhizes à arbuscules sur les racines adventives. Ce résultat est original car c'est la première fois que des nodules de tiges et des mycorhizes à arbuscules de racines adventives sont observés chez une légumineuse arborescente. L'inoculation avec la souche *Bradyrhizobium* sp. n'a pas d'effet bénéfique sur la biomasse totale du *Pterocarpus* notamment en milieu inondé malgré l'activité fixatrice d'azote (ARA) dans les nodules et l'acquisition de N dans les feuilles. La souche *G. intraradices* s'est révélée, par contre, efficace sur la biomasse totale des *P. officinalis* grâce à une colonisation racinaire tout à fait remarquable en milieu inondé. Cette colonisation racinaire par le champignon s'est aussi traduite par une augmentation du P dans les feuilles. L'oxygénation des parties submergées de la tige au travers des lenticelles, des racines adventives et des aérenchymes, pourrait favoriser la colonisation remarquable du système racinaire de *Pterocarpus* par le champignon mycorhizien à arbuscules. La double inoculation n'a cependant pas permis de mettre en évidence une synergie d'action des deux symbiotes sur la croissance et la fixation d'azote des *Pterocarpus*.

5. Conclusions et perspectives

L'aire de répartition de *P. officinalis* s'étend de l'Amérique continentale aux petites îles de la mer des Caraïbes. Du fait de leur isolement et de leur petite taille, les îles exacerbent les effets de dérive génétique, de dépression consanguine, et de différentiation entre les populations d'une même espèce (Barrett, 1996). Nos résultats expérimentaux ont confirmé les modèles théoriques en montrant d'une part, une forte différentiation entre les populations insulaires et, d'autre part, une faible diversité génétique dans les îles (Frankham, 1996). Ce type de structure de diversité génétique amplifie les risques d'extinction chez les espèces soumises à des pressions anthropiques (Frankham *et al*., 2002). Ceci pourrait être le cas de certaines populations de Dominique ou de Guadeloupe qui se limitent à quelques individus. De plus, les forts index de fixation trouvés dans certaines îles suggèrent la présence de consanguinité qu'il est nécessaire de lier à un risque inhérent d'extinction.

La diversité des populations de *Pterocarpus* comme celle des *Bradyrhizobium* associées est plus faible en milieu insulaire qu'en milieu continental. Les populations de *Pterocarpus* et

leurs bactéries auraient donc évolué en parallèle, conséquence possible de forces évolutives similaires (flux de gènes limités et fortes dérives génétiques attendues en milieu insulaire).

Malgré l'absence de spores de Glomales dans les sols marécageux, le potentiel mycorhizien reste relativement important entre 8 et 10‰ de sels le long du gradient de salinité. Les CMA sont présents dans ces sols sous forme de mycélium libre et/ou de fragments de racines mycorhizées. L'utilisation d'outils moléculaires (séquençage de l'ITS) sera nécessaire pour identifier les CMA à partir des fragments de mycorhizes.

P. officinalis a développé des mécanismes d'adaptation morphologique (lenticelles, racines adventives, aérenchymes) et physiologique (nodules de tige et , mycorhizes à arbuscules des racines adventives) en milieu inondé. La mycorhization a été efficace sur la croissance des *Pterocarpus* notamment en milieu inondé. Nous envisageons d'évaluer l'adaptation du *P. officinalis* et de ses symbiotes face aux deux contraintes (sel et inondation). Nous pourrons ainsi mieux appréhender le rôle des champignons mycorhiziens à arbuscules et des *Bradyrhizobium* sur la croissance de *P. officinalis* en vue de restaurer les forêts marécageuses dégradées et menacées d'extinction en Guadeloupe.

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ANNEXE

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PRIMER NOTE

Isolation and characterization of microsatellite markers in *Pterocarpus officinalis* Jacq.

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Abstract

The leguminous *Pterocarpus officinalis* Jacq. is one of the dominant freshwater wetland tree species in the Caribbean basin. Anthromorphic factors threaten to reduce its population. In order to investigate the genetic diversity and structure of this species, we developed eight pairs of primers for nuclear microsatellites. One hundred ninety-one individuals were analysed within nine Caribbean and continental populations. These loci were polymorphic in all the populations, with four to 20 alleles per locus. Significant Hardy–Weinberg deviation was detected and was interpreted as a result of Wahlund effect. These loci constitute a powerful tool to investigate the genetic patterns within populations of the swamp species *P. officinalis*.

Keywords: conservation, insularity, nuclear microsatellites, population genetic structure, *Pterocarpus officianalis* Jacq.

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Pterocarpus officinalis Jacq. is a leguminous forest tree species endemic to Caribbean swamp forests. Tropical marshy forests represent less than 2% of the Earth's emerged land. The major characteristic of this forest is temporal flooding with saltwater or freshwater during part of the year. These types of swamp forests are not well known both with regards to their structure and to their dynamics (Rivera-Occasion *et al.* 2002), in particular in the insular context (Guadeloupe and Martinique) and continental (French Guiana). *Pterocarpus officinalis* forests are present in different environments, within small islands or on the continent. To understand population dynamics and to implement a strategy for conservation of the species, it was decided to asses the genetic diversity and the structure of the populations on some Caribbean islands and on the continent. We chose nuclear microsatellite markers, which also allow the assessment of the mating system and the gene flow between populations. No nuclear microsatellite has been identified in *P. officinalis* species. In this paper, we report on the development of microsatellite primers from *P. officinalis* and their polymorphism in the Caribbean islands of Guadeloupe (4 populations), Puerto Rico (2 populations), Dominique (1 population), Martinique (1 population) and on the continent with French Guyana (1 population). Population samples were collected along rivers, in mountains and in wetland areas adjacent to mangrove. Twenty to 30 adult trees were randomly sampled and a total of 191 trees were selected. Two to five young to adult leaves were collected on each tree and dried in a plastic bag containing 10 g of silicagel. The leaves were then separated from silicagel and kept in a dry room at 20 °C.

Our genomic library was constructed using DNA sample from an individual that belongs to the Belle Plaine area population, which is located in the southeast of Guadeloupe.

Total DNA was extracted from 100 mg of dry leaf material using a mixed alkyltrimethylammonium bromide (MATAB) method derived from Bousquet *et al.* (1990), with one additional chloroform–isoamyl alcohol (24:1) extraction. Three micrograms of this purified total DNA was used to construct a $(GA)_n$ and $(CA)_n$ repeat-enriched genomic library,

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Table 1 Development, screening and polymorphism of eight nuclear microsatellites for *Pterocarpus officinalis* Jacq. Accession no. in the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database

Locus	Accession	Primer sequence	Motif	Allele size range (bp)	Annealing temperature	No.	n	H_E	H_O	*P* value
mPoCIRE01	AJ972377	5′ CTACCGTCCCCGATAAGCC 3′ 5′ GCGAGAGGCGTAGATCAAGC 3′*	$(TG)_{13}$	233–239	54 °C	4	157	0.49	0.50	1.10^{-1}
mPoCIRE04	AJ972488	5′ TCGCTATGACTAACATTTGC 3′ 5′ ATGGACACCGTAAAGAACC 3′*	$(TG)_{8}$	310–328	54 °C	7	138	0.45	0.19	1.10^{-4}
mPoCIRF08	AJ972489	5′ ACCAAATTGCTGTAGAATGAC 3′ 5′ ATCCGAAGATATTTCACTTACC 3′*	$(TG)_{18}$ $(AG)_{22}$	154–228	54 °C	20	140	0.84	0.56	1.10^{-4}
mPoCIRH02	AJ972490	5′ GCATCAATCCGAATGAGG 3′ 5′ TTTGAGGGGGTTGTAAAGG 3′*	$(CA)_{38}$	307–323	54 °C	5	26	0.57	0.31	1.10^{-3}
mPoCIRE09	AJ972491	5′ ACCGTGTCTTCGGATTCTGTTC 3′ 5′ TATCCTTGCATGCCCTTTGAG 3′*	$(CA)_{13}$	274–288	54 °C	8	155	0.64	0.33	1.10^{-5}
mPoCIRH08	AJ972492	5′ GTAAGCAGTTTTCTCTATTCTCTCTC 3′ 5′ CAACCGATCACTTCAACC 3′*	$(TC)_{17}$ $(AC)_{9}$	227–251	54 °C	7	158	0.3	0.18	1.10^{-5}
mPoCIRH07	AJ972494	5′ CACATCAAAATCATCAAATATACCC 3′ 5′ GACAAGCAAAACAGAAAGAAGC 3′*	$(CA)_{13}$	243–260	54 °C	4	62	0.59	0.22	1.10^{-3}
mPoCIRF03	AJ972493	5′ CTCCAGACAAACCAAAGGATC 3′ 5′ GGGAAAGAACCATCAAATGC 3′* * 5′ fluorescent-labelled primer: CACGACGTTGTAAAACGAC	$(CA)_{16}$ $(TA)_{7}$	125–159	54 °C	14	162	0.69	0.50	1.10^{-5}

n, number of individuals; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; H_O, observed heterozygosity; No., number of alleles; *P* values for the Hardy–Weinberg Equilibrium test, significance threshold adjusted using sequential Bonferroni correction: $P < 0.003$.

according to the Billote *et al.* (1999) protocol. A total of 42 clones were selected and sequenced using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems). Thirty-two of the sequenced clones contained a microsatellite region with at least four uninterrupted repeats. After discarding duplicates, hybrid clones and clones with the microsatellite region too close to the edge of the sequence, 16 sequences were found suitable for primer design and allowed a successful design using OLIGO EXPLORER software 1.1.0 (Teemu Kuulasma, 2000–2002; www.genelink.com), and were then tested for polymerase chain reaction (PCR) and polymorphism assessment. Eight loci were finally useful for our study, showing specific and polymorphic amplicons: mPoCIRE01, mPoCIRE04, mPoCIRF08, mPoCIRH02, mPoCIRE09, mPoCIRH08, mPoCIRF03 and mPoCIRH07 (Table 1).

PCR amplifications were performed using 10 μL total reaction volumes with 1 × *Taq* buffer (10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3; Roche), 200 μmol dNTP, 0.5 mmol each of forward and reverse primers, 0,1 μmol M13 IRD-labelled primer, 1 U *Taq* DNA polymerase and between 10 and 30 ng of genomic DNA template. The amplifications were carried out with a Robocycler gradient 96 (Stratagene) thermal cycler under the following conditions: an initial 4 min denaturation step at 94 °C followed by 36 cycles consisting in 30 s at 92 °C, 1 min at 54 °C, 45 s at 72 °C and a final extension step at 72 °C for 5 min. Following amplification, 3 μL of loading dye (100% formamide) was added to each reaction. For final screening, the microsatellites were detected on a LI-COR 4200 sequencer with a 7% polyacrylamide (Long Ranger) gels.

The number of alleles found ranged from four to 20 per locus and was substantially higher in the continent than in the Islands. Microsatellite genotypes were tested for linkage disequilibria for all pairs of loci within each population using Fisher's exact test. Unbiased exact *P* value estimates were obtained by the Markov chain method computed by GENEPOP (Raymond & Rousset 1995) with 5000 permutations, and significance was adjusted using the Bonferroni correction. Loci were at linkage equilibrium in all of the populations we studied.

Significant deviations from Hardy–Weinberg equilibrium were detected for all loci using GENEPOP software. In all cases, the observed frequency of heterozygotes was lower than the expected frequency (*P* values < 0.003, significance threshold adjusted with the Bonferroni procedure (Rice 1989)) (Table 1). The use of MICRO-CHECKER software (Van Oosterhout *et al.* 2004) suggested the presence of null alleles in the total population, which could explain this deviation from Hardy–Weinberg equilibrium. However, this could be interpreted as the result of a Wahlund effect because the populations were structured in several subpopulations. Actually, most of the loci tend to have heterozygote deficits, which speak for a demographic effect. These loci provide the first set of microsatellite markers derived directly from the *P. officinalis* Jacq. genome.

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Arbuscular mycorrhizal soil infectivity in a stand of the wetland tree *Pterocarpus officinalis* along a salinity gradient

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Abstract

Pterocarpus officinalis (Jacq.) is the dominant wetland legume tree species of the seasonally flooded swamp forests in Guadeloupe, Lesser Antilles. This tree is periodically exposed to saline and flooded soil conditions. We examined mycorrhizal soil infectivity (most probable number (MPN) values) and arbuscular mycorrhizal (AM) colonization of *P. officinalis* along the salinity gradient where the salt levels ranged from 26 to 2‰ and from 22 to 5‰ at the end of the dry and wet season, respectively. MPN values were higher in the dry season than in the wet season. They decreased when the salt levels increased whatever the season. AM colonization of *P. officinalis* was well developed (up to 50%) only within the low salinity levels (below 10‰) whatever the season. No spores were found in soil cores, suggesting that propagules were only mycelium pieces and/or root fragments of colonized *Pterocarpus* roots. These AM fungi may be adapted to salt stress and explain the maintenance of the high mycorrhizal inoculum potential in the *P. officinalis* swamp forest.

Keywords: Arbuscular mycorrhizal colonization; Swamp forest; Salinity; Flooding; Guadeloupe

1. Introduction

Arbuscular mycorrhizal fungi (AMF) in wetland habitats are exposed periodically to anaerobic soils and high soil salinity (Bohrer et al., 2004; Carvalho et al., 2004). Since AMF require oxygen to thrive, stressful environments regularly flooded with salt water may be detrimental for their survival and infectivity. Nevertheless, some AMF are able to persist in flooded soils and to colonize wetland plants (Khan, 1993; Miller and Bever, 1999; Turner et al., 2000; Landwehr et al., 2002). More than 50% of the plant's population were colonized by AMF in some wetlands conditions (Ragupathy et al., 1990). One species, *Glomus geosporum*, is usually dominant in European salt marshes (Landwehr et al., 2002; Carvalho et al., 2004). These studies suggest fungal adaptation to salt and flood conditions. In this respect, AMF possess propagules allowing long-term survival in soils and immediate opportunistic root colonization (Smith and Read, 1997). Spores, infected root fragments and extraradical mycelia are the main sources of inoculum potential in soils contributing to the infectivity of plants. The relative contribution of each type of propagules to plant root colonization is difficult to establish. Depending on fungal species, spore production and germination were affected by salinity and soil water levels (Miller and Bever, 1999; Carvalho et al., 2004). Le Tacon et al. (1986) reported that anaerobic conditions inhibited the germination of spores of *Glomus mosseae*, but that this effect was reversible upon exposure of the spores to air. The identity of the host plant was also known to be an important factor affecting density of AM propagules and sporulation (Smith and Read, 1997). However, extraradical mycelia and root fragments seemed to be relatively more important than spores for the initiation of plant colonization in wetlands, salt mashes and aquatic systems (Brown and Bledsoe, 1996; Carvalho et al., 2004).

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In the present work, we studied the AM inoculum potential in swamp soils from a monospecific stand of the wetland legume tree *Pterocarpus officinalis*. This tree is useful for the study of AM soil infectivity because it grows along a gradient of salinity. This allowed us also to determine the effect of salinity on AM colonization, while keeping the host plant constant.

2. Materials and methods

2.1. Study site

The *Pterocarpus* swamp forest covers 2600 ha of freshwater, coastal wetlands in Guadeloupe (Imbert et al., 2000). It is found mainly around the bay of the Grand cul-de-sac Marin, behind mangrove areas. The study site is located on the eastern side of the bay, in the Abymes coastal plain (61°30′N, 16°10′W). At this site, the swamp forest is separated from the mangrove forest by a narrow strip (ca. 50 m-wide) of mainly herbaceous vegetation, dominated by the fern *Acrostichum danaefolium* and the liana *Rhabdadenia biflora*. Some stunted *Pterocarpus* trees, mostly dead, occur inside this ecotope zone. From the swamp forest edge inwards, tree height gradually rises from about 5 to 20 m and over. As the canopy rises, stem density decreases, whereas basal area increases. *P. officinalis* is the only tree species contributing to the forest canopy. Understorey species, like *Coccoloba venosa* and *Montrichardia arborescens*, are few and far between. The forest is flooded most of the year, depending on the duration of the rainy season. Water level may vary from place to place, due to *Pterocarpus* buttresses that create low mounds. The soil is clayey, soft and brownish in the upper 30 cm, denser and greyer below.

2.2. Sampling and measurements

The investigations were conducted along a transect starting from the seaward edge toward the inner part of the swamp forest. Sampling was made on three mature trees of *P. officinalis* in six plots (3 m × 3 m) along a part of the transect that spanned an existing salinity gradient. The length of this part of the transect was approximately 170 m. Water-table level was assessed around each sampled tree by measuring water depth in comparison with ground surface. Pore-water salinity at 20 cm below ground level and salinity of above-ground standing water were measured using an ATAGO ATC-S, temperature-compensated hand refractometer (ATAGO Inc., Bellevue, WA, USA). Salinity and water depth measurements were made at the end of the dry and wet season.

For each plot, five soil cores (20 cm in depth; 7 cm in diameter) were taken around each mature tree, pooled per soil salinity level and stored at room temperature (20 °C). The method chosen for mycorrhizal soil infectivity determination was the most probable number (MPN) method (Porter, 1979). The method involves cultivation of a test plant having a high mycorrhizal dependency (Plenchette et al., 1983) on a range of successive dilutions of the soil to be tested. Soil samples were air dried and sieved (2 mm) prior to disinfect a sub sample of each by autoclaving (115 °C, 45 min). The remaining humidity was determined. Then, for each sample, non-disinfected (ND) and disinfected (D) soils were mixed together to obtain the following range of dilutions: 1, 1/4, 1/16, 1/64, 1/256 and 1/1024 (ND/D; w/w). Five replicates were made for each dilution. Soil (100 g) were placed in small pots then planted with a 2-week-old seedling of millet (*Pennisetum americanum* L.) for a 6-week growing period. Pots were placed in a controlled environment (24 °C day/18 °C night; relative humidity 80%; light intensity 112 μmol m^{-2} s^{-1}). After 6 weeks the entire root system of each plant was gently washed, cleared and stained (Phillips and Hayman, 1970). Observation of colonization was made under a dissecting microscope 50×. One infection point was considered as sufficient to state that the plant was colonized. Calculation of MPN values were made as described by Sieverding (1991) according to Fisher and Yates (1948) and expressed per 100 g of dried soil.

The fine roots were randomly collected for each mature tree of *P. officinalis* (three replicates per tree), gently washed, cleared and stained (Phillips and Hayman, 1970). Roots were then cut into 1 cm pieces, mixed and placed on slides for microscopic observations at 250× magnification (Brundrett et al., 1985). One hundred root pieces were observed per plant. The extent of AM colonization was expressed as a percentage of the number of mycorrhizal root pieces/number of non-mycorrhizal root pieces. Data of mycorrhizal colonization were subjected to one-way analysis of variance, and mean values were compared using Newman–Keuls multiple range tests (Gagnon et al., 1989). Data of mycorrhizal colonization were transformed by arcsin (square root) before analysis.

3. Results

The salinity gradient ranged from 26 to 2‰ and from 22 to 5‰ at the end of the dry and wet season, respectively (Tables 1

Table 1
Soil salinity, mycorrhizal soil infectivity (MPN and confident limits) and mycorrhizal colonization of *P. officinalis* at the end of the dry season

Plots	Salinity ‰ (0–20 cm)	Water depth (cm)	MPN* (100 g)	Confident limits $p < 0.05$	Colonization** (%)
1	26	4	75 a	35–160	14.5 a
2	20	3	150 a	70–320	29.4 b
3	15.5	21	560 b	262–1196	47.5 c
4	10	−12	2400 bc	1123–5126	66.0 d
5	3	−3	3402 c	1592–7266	76.9 e
6	2	−7	3800 c	1779–8166	82.8 e

* Values followed by the same letters are not significantly different (confident limits, $p < 0.05$).
** Values followed by the same letters are not significantly different (Newman–Keuls, $p < 0.05$).

Table 2
Soil salinity, mycorrhizal soil infectivity (MPN and confident limits) and mycorrhizal colonization of *P. officinalis* at the end of the wet season

Plots	Salinity (‰) (0–20 cm)	Water depth (cm)	MPN* (100 g)	Confident limits $p < 0.05$	Colonization** (%)
1	22	16	20 a	9–42	22.1 a
2	15	15	31 a	15–67	40.3 b
3	13.5	33	90 bc	42–191	48.8 b
4	8	0	1157 d	542–2472	65.1 c
5	3	9	688 d	322–1469	68.9 c
6	5	5	301 cd	141–644	75.1 c

* Values followed by the same letters are not significantly different (confident limits, $p < 0.05$).
** Values followed by the same letters are not significantly different (Newman–Keuls, $p < 0.05$).

Table 3
Correlation coefficients (r) between mycorrhizal soil infectivity (MPN values), soil salinity (S‰) and mycorrhizal colonization of roots (% AM colonization)

	Dry season			Wet season		
	MPN	S	AM (%)	MPN	S	AM (%)
MPN		−0.96*	0.95*		−0.86*	0.75*
S			−0.99*			−0.97*

* Significant ($p < 0.05$).

and 2). There was not an evident flooding gradient in wet and dry seasons in the *P. officinalis* swamp forest (Tables 1 and 2).

Spores were not found in the soil samples collected during the dry and wet seasons (Tables 1 and 2). Nevertheless, AMF were present in the root samples of *P. officinalis*, whatever the salt level (Tables 1 and 2). The higher AM colonization (82–75%) was recorded for the lowest salt level (2–5‰) (Tables 1 and 2). AM colonization was greater to 50% until the salt levels of 8 and 10‰ were reached in wet and dry seasons, respectively. MPN values due to mycelium and root fragments also decreased as salt level increased (Tables 1 and 2). However, MPN values were higher in the dry season than in the wet season.

Significant negative correlations were obtained between the salt levels and the MPN values both in wet and dry seasons (Table 3). MPN values and AMF colonization were positively correlated in dry season (Table 3). Whatever the season, colonization percentage was negatively correlated with salinity level (Table 3, Fig. 1).

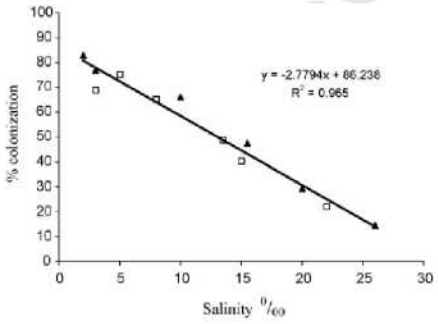

Fig. 1. Relationship between salinity level and development of AM colonization. (□) Dry season; (▲) wet season.

4. Discussion

Salinity has been shown to be an important factor influencing AM colonization of *P. officinalis* and fungal inoculum potential in swamp soils. AM colonization was indicated by the presence of aseptate hyphal coils and vesicles as *Paris*-type mycorrhizas (Bâ et al., 2004). Surprisingly, no spores were found in sieved soil samples collected during the dry and wet seasons. Since spores were absent, we conclude that the infection units and colonization of roots were due to mycelium and/or root fragments. We do not know the relative proportion of infection units and AM colonization derived from mycelium or root fragments. Furthermore, it is not clear whether the absence of spores in swamp soils was due to the toxic levels of minerals and/or to variations of depth water in dry and wet seasons. Some studies indicate that water depth is an important factor determining the distribution of spore species along a dry to wet gradient (Miller and Bever, 1999). The overall trend was for fewer spore species in wetter sites than in drier sites. Other studies suggest that soils from salt marshes contain spores of AMF in high numbers, whereas reduction of spore germination at water levels above field capacity may be related to the low tolerance of AMF to hypoxic conditions (Landwehr et al., 2002; Carvalho et al., 2004).

We found evidence for potential adaptation of native AMF to salt swamp soils and for the ability of AM propagules to spread into the roots. Indeed, AMF colonization was greater to 50% until the salt level of 8‰ was reached in dry and wet seasons and decreased in both seasons as salt level increased. Since AM colonization was already recorded on wetlands plants (Bohrer et al., 2004), some results were unexpected at very high salinity levels (Brown and Bledsoe, 1996). Our results suggest that AMF were well adapted to stressful salt swamp soils. The absence of spore in sieved swamp soils did not permit conclusions as to the taxonomy of these AMF. PCR with taxon-specific primers have been used to identify AMF within colonized roots (Landwehr et al., 2002). We plan to incorporate such techniques in our future work.

AM colonization did not vary significantly from wet to dry season, whereas the MPN values did. One possible explanation is that fungi may endure prolonged exposure to salt and flooding by spreading inside the *Pterocarpus* roots, whereas extraradical mycelium and root fragments in soils may not. In this respect, roots of *P. officinalis* would be well aerated due to aerenchymatous tissue, adventive roots and buttress lenticels.

Brown and Bledsoe (1996) observed AMF in the aerenchymatous tissue of salt marsh plants, suggesting that AMF are adapted to life in oxygen-deficient soils. MPN values were mainly higher than those recorded in other tropical soils, i.e., 10 propagules/100 g for a lowland wet forest in Costa Rica (Fisher et al., 1994) or 1–100 propagules/100 g for soils used for intensive banana cultivation in Martinique (Declerck et al., 1999). This suggests that *P. officinalis* may be considered as a high mycorrhizal dependent tree which favor AM fungal development. The high mycorrhizal soil infectivity could be also a real potential for the traditional culture of taro in the *Pterocarpus* swamp forests (Saur and Imbert, 2003). From an ecological point of view, AM soil infectivity and seasonal dynamics of AM colonization should be considered further to better assess the role and the distribution of AMF in the *Pterocarpus* swamp forests.

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Mycorrhiza (submitted)

Nodulation and arbuscular mycorrhizal colonization improve flooding tolerance in *Pterocarpus officinalis* Jacq. seedlings

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Abstract

Pterocarpus officinalis (Jacq.) seedlings inoculated with the strain of *Bradyrhizobium* sp. (UAG 11A) and the arbuscular mycorrhizal (AM) fungus, *Glomus intraradices*, were grown under stem-flooded or non-flooded conditions for thirteen weeks, following 4 weeks of non-flooded pre-treatment under greenhouse condition. Flooding of *P. officinalis* seedlings induced several morphological and physiological adaptive mechanisms including formation of hypertrophied lenticels, aerenchyma tissue and production of adventitious roots on submerged portions of the stem. Flooding resulted also in an increase in collar diameter, leaf, stem, root and to total dry weights, regardless of inoculation. We report here the novel occurrence of nodules connected vascularly to the stem as well as nodule and arbuscular mycorrhiza on adventitious roots of *P. officinalis* seedlings. Nodules formed on submerged stems and adventitious roots of flooded seedlings, and were able to fix N_2 (3-4 μmoles C_2H_4/h/g nodule dry weight). Root nodules formed also on both non-flooded and flooded seedlings, and were capable of fixing N_2 (7-9 μmoles C_2H_4/h/g nodule dry weight). Root nodules appeared more efficient fixing N_2 than were stem nodules. Beneficial effect of nodulation in terms of total dry weight and N acquisition in leaves was particularly noted in seedlings growing under flooding conditions. Still under flooding arbuscular mycorrhizas was well developed on root systems and adventitious roots as compared with inoculated root systems under non-flooding condition. Therefore, the arbuscular mycorrhizas contributed noteworthy to the flood-tolerance of *P. officinalis* seedlings by improving plant growth and P acquisition in leaves. Nevertheless, there was no additive effect of arbuscular mycorrhiza and nodulation on plant growth and nutrition whatever the water level. The results suggest that the development of adventitious roots, aerenchyma tissue and hypertrophied lenticels may play a major role in flooded tolerance of *P. officinalis* symbiosis by increasing oxygen diffusion to the submerged part of the stem and root zone, and therefore contribute to plant growth and nutrition.

Keywords : Nitrogen fixation, *Bradyrhizobium*, Stem nodules, *Glomus intraradices*, Mycorrhizal adventitious roots.

Introduction

Much of the wetlands are subject to flooding due to the presence of shallow water tables and a decrease of the surface water infiltration (Barrett-Lennard 2003). Flooding causes a condition of hypoxia or anoxia in soils because of the low solubility and diffusivity of oxygen in water and the rapid use of dissolved oxygen by microorganisms and roots. Therefore, there is a decrease in the mineralization of organic matter and an increase in denitrification (Barrios and Herrera 1993). Moreover, the heavily leached soils, brought about by seasonal flooding, contribute in the shortage of available N and P. In this context, a number of plants are nodulated legumes (Walter and Bien 1989; Moreira et al. 1992; Loureiro et al. 1995; Saur et al. 1998; James et al. 2001; Koponen et al. 2003) that can be also associated with arbuscular mycorrhizal (AM) fungi (Sanchez-Diaz et al. 1990; Carvalho et al. 2003; Bâ et al. 2004). While many studies have well documented the occurrence of AM fungi in wetland (Bohrer et al. 2004; Carvalho et al. 2004 ; Saint-Etienne et al. 2006), their importance and function are limited and often contradictory. It is not clear if flooding has an effect on AM association that can be generalized, as some results show an improvement in growth and P nutrition over non-colonized plants (Wigand and Stevenson 1997 ; Osundina 1998 ; Miller and Sharitz 2000), some have shown a decrease (Steven et al. 2002), and others did not detect a clear relationship (Hartmond et al. 1987). Nevertheless, the lack of external input of N in ecosystem increases the demand for biological nitrogen fixation (Dommergues et al. 1999). Indeed, it has already been shown that nodulated legumes contribute significantly to the N-balance of tropical wetlands and rainforests (Roggy et al. 1999 a,b ; Koponen et al. 2003; Diabaté et al. 2005). Legumes generally require P from mycorrhiza for their nodule formation, nitrogen fixation and growth (Dommergues et al. 1999 ; Vance 2001). However, little is known about the interactions between AM fungi and nitrogen fixing bacteria in legumes growing in wetland ecosystems.

Pterocarpus officinalis (Jacq.) (Fabaceae) is the dominant wetland tree species of the seasonally flooded swamp forests in the Caribbean basin (Eusse and Aide 1999 ; Imbert et al. 2000 ; Muller et al. 2006). It covers large areas of the coastal floodplain, and individual trees occur along rivers and in the mountains. The establishment and population maintenance of *P. officinalis* are affected by the variation in salinity and hydrology as well as differences in soil microtopography in the swamp forests (Alvarez-Lopez 1990; Eusse and Aide 1999). This tropical wetland tree species forms bradyrhizobial nodules and arbuscular mycorrhizas on lateral roots of buttresses both above and below the water table (Saur et al. 1998; Bâ et al. 2004; Saint-Etienne et al. 2006). Alvarez-Lopez (1990) suggested that *P. officinalis* cannot establish seedlings under flooding conditions, because rooting of germinated fruit did not occur in water over 3-4 cm deep. If flooding persists, only germinated seeds, transported by water movement to more topographic elevation, may survived (Alvarez-Lopez 1990). We hypothesized that nodulation and arbuscular mycorrhiza could contribute to *P. officinalis* seedlings performance under flooding. Two questions are addressed in the present study: (1) do *P. officinalis* seedlings can adapt to flooding? (2) do arbuscular mycorrhiza and N_2-fixing nodules increase the performance of *P. officinalis* seedlings under flooding?

Materials and methods

Bacterial and fungal inocula

Bradyrhizobium sp. (UAG 11 A) strain was isolated from a root nodule collected during the dry season from mature *P. officinalis* tree growing in the swamp forest of Port Louis in Guadeloupe, Lesser Antilles (Bâ et al. 2004). The bacterial inoculant consisted in a 10-day-old pure culture grown on liquid yeast extract-mannitol medium (Vincent 1970).

The AM fungus, *Glomus intraradices* Schenck & Smith (DAOM 181602, Ottawa, Agricultural Herbarium), was propagated on leeks (*Allium porrum* L.) growing on Terragreen™ substrate for 12 weeks on a calcined clay (Oil-Dri US-special Ty/IIIR, Oil-Dri Company, Chicago, USA) (Plenchette et al. 1996) under greenhouse conditions. The leek plants were uprooted, their roots gently washed and cut into pieces 0.5 cm long pieces. Non-AM leek roots, prepared as above, were used for the control treatment.

Seed germination

Pods of *P. officinalis* were collected along river in the swamp forest of Grande Ravine in Guadeloupe. Pods were shelled and seeds were surface sterilized with 3% sodium hypochlorite (w/w) for 10 min. They were then rinsed several times in sterile water and germinated in sterile vermiculite at 25°C in the dark. The germinated seeds were used when tap roots were 2-3 cm long.

Experimental design

The substrate used is a mixture of heat-sterilized pouzzolane (crushed volcanic rock with particle size average 2 mm) and vermiculite (4:1; v/v). The nutrient content of the heat-sterilized crushed volcanic rock was as follow: 4.28 K, 15.67 Na, 6.36 Ca, 4.99 Mg, 1.26 NH_4^+, 2.75 NO_3^- (H_2O-extractable) ppm, 0.12 ppm Olsen-P, pH (H_2O) = 8.41, pH (KCl)= 7.2, 0.11 g/l total salt and electrical conductivity 0.036 mS/cm. Black pots (22 cm deep, 9 cm diameter) were filled with the substrate to within 4 cm of the rim. Soil leakage was prevented by placing a wad of polyester fiber at the bottom of each pot.

The seedlings were transplanted in pots and inoculated with *Bradyrhizobium* sp. alone, *G. intraradices* alone or with both microbial partners. Before sowing, 0.35 g of fresh leek roots colonized by *G. intraradices* (*Gi*) (with about 250 vesicules cm^{-1}) was inoculated in a hole of the substrate close to the *P. officinalis* root system, or 0.35 g of non-AM leek roots for the controls. Bradyrhizobial inoculation was then performed on seedlings by spreading 5 ml (10^9 bacterial cells ml^{-1}) of a suspension of *Bradyrhizobium* sp. (*Br*) on tap root or 5 ml of the culture medium without bacteria for the controls. All the plants were grown under well-watered conditions without nutrients for 4 weeks in a greenhouse receiving approximately 30% of the photosynthetic active radiation between February and June 2003, at 25°C-35°C with a day-length of about 12 h. After 4 weeks, flooding was imposed to half of the pots. Seedlings were periodically flooded by marking up water level to 3 cm above the soil surface to replace evapored and/or transpired water in pots without drainage holes. Non-flooded seedlings were watered near the field capacity in pots with drainage holes. The pots were arranged in a completely random 4 x 2 factorial design comprising eight treatments: (1) flooded *Gi*, (2) flooded *Br*, (3) flooded *Gi* plus *Br*, (4) flooded non-inoculated, (5) non-

flooded *Gi*, (6) non-flooded *Br*, (7) non-flooded *Gi* plus *Br*, (8) non-flooded, non-inoculated. Each treatment consisted of ten replicates. The plants were supplied each month with 50 ml of Long Ashton's nutrient solution (Hewitt 1966) without P and N. Treatments were imposed for 13 weeks after flooding was established.

Seedling measurements and nutrient analysis

Thirteen weeks after flooding, the number of adventitious roots was determined by counting the roots emerging from the epicotyl. Nodules were collected separately from root and stem (including adventitious root nodules) and counted.

The acetylene reduction assay (ARA) was made on ten freshly detached nodules randomly collected on roots and stems. Nodules were immediately sealed in serum-capped vials, filled with 10% acetylene (C_2H_2) in air, and incubated at laboratory temperature for 1 h. Then, gas aliquots of 10 ml were removed from the vial and injected into a "Vacutainer" for storage. Ethylene (C_2H_4) and acetylene concentrations of the sample were analysed using flame ionization in a Hewlett Packard 5890 II gas chromatography. The ARA results were considered as positive when the mean C_2H_4 concentration after incubation with nodule was more than 1 µmole/g of dry nodule/h. Control root samples without nodules did not show concentration above this value. Nodules were dried at 80°C for 7 days and weighed after each assay.

Height, collar diameter and dry weight (7 days at 80°C) of leaves, shoots, roots, adventitious roots and the remaining stem and root nodules were evaluated. AM fungal colonization of roots and adventitious roots were also measured. The roots were randomly collected for each seedling, gently washed, cleared and stained (Phillips and Hayman 1970). Roots were then cut into 1 cm pieces, mixed and placed on slides for microscopic observations at 250 x magnification (Brundrett et al. 1985). Hundred root pieces were observed per plant. The extent of AM colonization was expressed as a percentage of number of mycorrhizal root pieces / number of non-mycorrhizal root pieces.

After drying, ground leaf samples were mineralized through heating at 500°C and digested in chloric acid for determination of N and P. The total N contents of leaves were estimated using a Technicon autoanalyser. P was determined by colorimetry with chain in continuous flow (Technicon) according to Novozamsky et al. (1983).

Microscopy

Nodule-bearing stem portions were cut from plants, fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C, and rinsed in the same buffer. Thick sections (100µm thick) of whole nodules plus the bearing stem were realized using a Vibratome (Leica, France), cleared in sodium hypochloride and observed under an Olympus SZH stereomicroscope.

For thin sections, samples were post-fixed for 1 h in 2% osmium tetroxide and rinsed in distilled water. They were deshydrated through an ethanol series followed by three washes in pure ethanol, infiltrated by an ethanol-Spurr's resin series and then embedded in 100% Spurr's resin. Polymerization took place at 70°C for 48 h. Approximately 0.5-1 mm thick sections were stained with 0.05% toluidine blue 0 in 1% borax and examined using a Leitz Ortholux light microscope.

Statistical analysis

All data were subjected to two-way analysis of variance, and mean values were compared using Newman-Keuls multiple range test (Gagnon et al. 1989). Mean number of nodules and percentages of mycorrhizal colonization were calculated from arc sin (square root) transformed data.

Results

After thirteen weeks of flooding, hypertrophied lenticels and aerenchyma (not shown) tissue were observed on the submerged portion of the stem above the soil line (Fig. 1A). Few thick, white, elongated and branched adventitious roots had grown from stems (Fig. 1A).

Serial thick sections of stem nodules (Fig 1B and 1C) clearly evidence the direct link of the peripheral nodular vascularization to the stem vascular bundles. Numerous fixation zones are visible in the nodule (Fig 1D), with cells densely filled with bacteroids (Fig 1E). The histological organization of stem nodules is similar to that of aeschynomenoid type of nodules according to Corby (1988), where an intercellular mode of entry takes place at bases of lateral or adventitious roots (Goormachtig et al. 2004).

Nearly all inoculated treatments produced significant effects on growth and mineral acquisition traits (Table 1). Water level treatments had no significant effect for root and root nodule dry matter, number of root nodules, nitrogen fixation in root nodules, N and P in leaves. Interactions between inoculated status and water level were significant for height, stem dry matter, number of root and stem nodules, stem nodule dry matter, AM colonization and nitrogen fixation in stem nodules.

A few nodules were observed on roots of control plants (Table 3). However, they did not fix nitrogen efficiently when compared with inoculated treatments (Table 4). Number, dry weight and nitrogen fixation of root nodules were nearly the same in plants inoculated by *Bradyrhizobium* sp. under both flooding and unflooding (Tables 3 and 4).

No AM fungal colonization was noted in roots and adventitious roots of control plants (Table 3). AM structures (vesicles and hyphal coils) were found at all levels of water. *Pterocarpus* plants grown under flooding had relatively high AM fungal colonization as compared to inoculated plants under unflooding. Elongated adventitious roots were also well colonized by *G. intraradices* as they penetrated into the soil (Table 3).

Inoculated treatments did not increase number and dry weight of adventitious roots (Tables 1 and 2). Collar diameter, leaf, stem and total dry weights were generally higher under flooding than unflooding (Table 2). *Bradyrhizobium* alone enhanced total biomass only under unflooding. By contrast, *G. intraradices* alone stimulated total biomass under both flooding and unflooding. There was no additive effect of *Bradyrhizobium* and *G. intraradices* on plant growth. However, *G. intraradices* alone or with *Bradyrhizobium* appeared to be more effective in increasing total biomass of *Pterocarpus* seedlings particularly under flooding.

The nitrogenase activity was lower in stem nodules than in root nodules of seedlings inoculated by *Bradyrhizobium* under flooding (Table 4). There was no difference in nitrogen-fixing root nodules of inoculated *P. officinalis* seedlings by *Bradyrhizobium* both under

flooding and unflooding (Table 4). The concentrations of N in leaves of plants inoculated by *Bradyrhizobium* compared with non-inoculated plants showed that nitrogen-fixing root nodules were similarly efficient both under flooding and unflooding. Moreover, *G. intraradices* significantly contributed to P acquisition in seedlings under flooding or unflooding. Nevertheless, P acquisition did not improve nitrogen-fixing stem and root nodules of *Pterocarpus* seedlings (Table 4).

Discussion

Flooding induced several physiological and morphological changes in *P. officinalis* seedlings including formation of hypertrophied lenticels, aerenchyma tissue and adventitious roots on submerged portions of the stem. Flooded plants grew overall better than non-flooded ones regardless of inoculation. These results suggest that *P. officinalis* seedlings can endure thirteen weeks flooding by developing some adaptive mechanisms. The high flooding tolerance of some trees has been mainly attributed to the production of adventitious roots that play a major role in water absorption and stomatal opening (Gomes and Kozlowski 1980; Crawford 1982; Liao and Lin 2001; Entry et al. 2002; Carter et al., 2005). However, we did not measure the activity of adventitious roots in the present study.

Flooding induced also nodules both on adventitious roots arising from stems as well as on the stems themselves. Stem nodules formed only on submerged parts of flooded seedlings to within 1 cm of the soil surface, and flooding appeared essential for their formation. Adventitious root nodules formed along stem below the water line. These observations, combined with observations made on seedlings in the swamp forests of Guadeloupe (Bâ unpubl. data), suggest that stem nodules are formed and grow on *P. officinalis* seedlings only under flooding. The root nodules developed and fixed N_2 similarly under both flooding and non-flooding conditions. Therefore, flooding did not affect root nodule formation and N_2 fixation as shown on *Discolobium pulchellum* (Loureiro et al. 1994). There were also more root nodules than stem nodules. This could be explained why nitrogenase activity of root nodules was greater than that of corresponding stem nodules on *P. officinalis* seedlings. There was also a significant increase of N in leaves of both flooded and non-flooded inoculated plants by *Bradyrhizobium*, resulting to an increase of the N_2-fixing of stem and root nodules. Nevertheless, under flooded conditions, N acquisition did not enhance total dry weight when compared with non-inoculated controls. One possible explanation for this may be the nitrogenase activity that we detected in root nodules of controls. However, N_2 fixation appeared to be similar to that in swamp forests (Saur et al. 1998), but very low compared to that of hydrophytes such as *Aeschynomene*, *Sesbania*, and *Discolobium* (Dreyfus and Dommergues 1981; Alazard 1985; Loureiro et al. 1994).

We report here the novel occurrence of stem nodules in *P. officinalis* seedlings. It is clear that, in flooding condition, *P. officinalis* seedlings formed true stem nodules, *i.e.* connected vascularly to the stem and not to adventitious roots arising from the stem. In this respect, James et al. (1992) have suggested that only nodules with vascular connection to the submerged or unsubmerged stem itself should be considered as stem nodules. We have also shown that the stem nodules require flooding for their formation. However, once formed, we don't know whether stem nodules were able to fix nitrogen under non-flooded conditions as shown on the legume *Aeschynomene fluminensis* (Loureiro et al. 1995). Stem nodulation is a relatively rare structure that has been confirmed only in some nodulated hydrophytes such as *Aeschynomene*, *Sesbania*, *Discolobium* and *Vigna* growing on seasonally or permanently flooded wetlands in Africa and South America (Dreyfus and Dommergues 1981; Alazard

1985; Loureiro et al. 1995; James et al. 2001). *P. officinalis* seedlings develop also nodules attached to adventitious roots emerging from stem below the water line. We don't know whether the nodules on adventitious roots of *P. officinalis* seedlings have an obligate need for submergence and thus senesce very quickly on exposure to air. However, the adventitious root nodules (not shown) are not considered as true stem nodules as they are anatomically more similar to root nodules according to the criteria defined by James et al. (1992). Aerial nodules were similarly attached to adventitious roots of the legume, *Pentaclethra macroloba*, in a lowland tropical rainforest swamp in Costa Rica (Walter and Bien 1989). Prin et al. (1991) discovered also the aerial nodulation on the trunks of *Casuarina cunninghamiana* in the North of Reunion island.

In our study, arbuscular mycorrhiza and root nodules were formed during the first 4 weeks when seedlings were watered near the field capacity. This means that we have assessed in part the properties of mycorrhizas and nodules already established and those of new arbuscular mycorrhizas and nodules formed under flooding. These symbiotic associations appeared to have been maintained after the substrate was flooded. Since AM fungi require oxygen to thrive, stressful regularly flooded environments may be detrimental to their survival and infectivity (Smith and Read 1997). Nevertheless, evidence that the AM fungus remained viable under flooding was provided by the increase of the proportion of colonized root and P acquisition in leaves of *P. officinalis* seedlings. The increased in AM colonization with rising water level in seedlings suggests a direct relation between flooding and AM colonization. This can be explained why the development of lenticels, aerenchymatous tissue and adventitious roots on the submerged part of the stem could facilitate oxygen transport to support newly colonized roots. This result is in agreement with what was reported for salt marsh plants colonized by AM fungi (Brown and Bledsoe 1996 ; Carter et al. 2005). Mycorrhizal *Casuarina equisetifolia* seedlings better adapted to flooding than non-inoculated seedlings because the greater development of adventitious roots and lenticels increased oxygen availability and therefore AM colonization of plants (Osundina 1998). Some studies suggested also that once AM colonization has taken place, the AM association can endure prolonged exposure to flooding (Miller and Sharitz 2000).

To conclude, this study showed that infectivity and activity of the nodulated and AM plants seem to be dependent on soil flooding. Furthermore, it provides supporting evidence that AM colonization by *G. intraradices* contributed substantially to the flooded tolerance of *P. officinalis* seedlings. This could be due to the increasing O_2 diffusion through the greater development of adventious roots, aerenchymatous tissue and hypertrophied lenticels on the root zone and submerged part of the stem. It is therefore possible that AM colonization and nodulation may contribute to the establishment of *P. officinalis* along a wider range of soil flooding levels in swamp forests. However, further experimental investigations should be done to understand the mechanims by which AMF increases AM colonization of *P. officinalis* seedlings in flooding and its adaptive significance.

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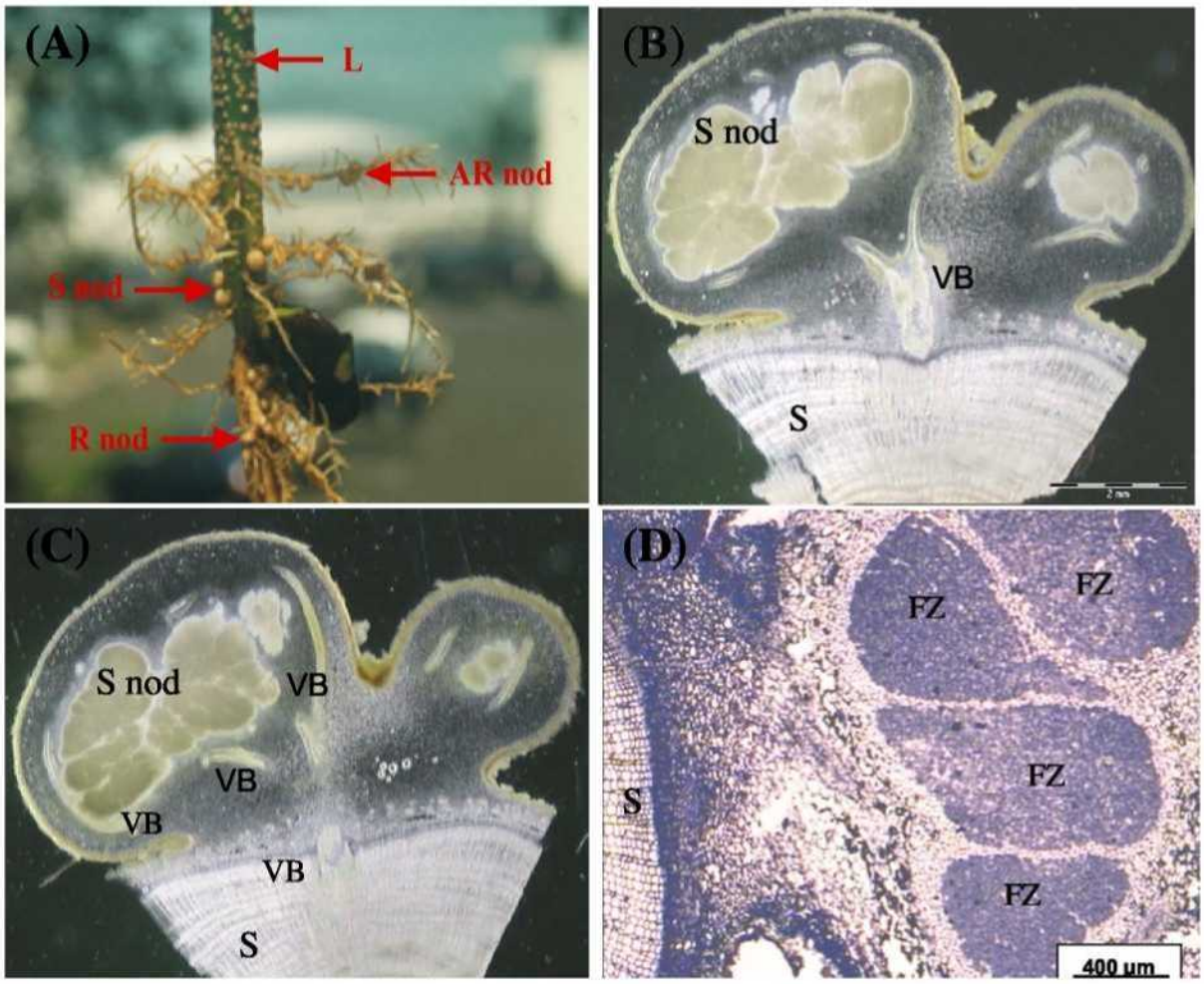

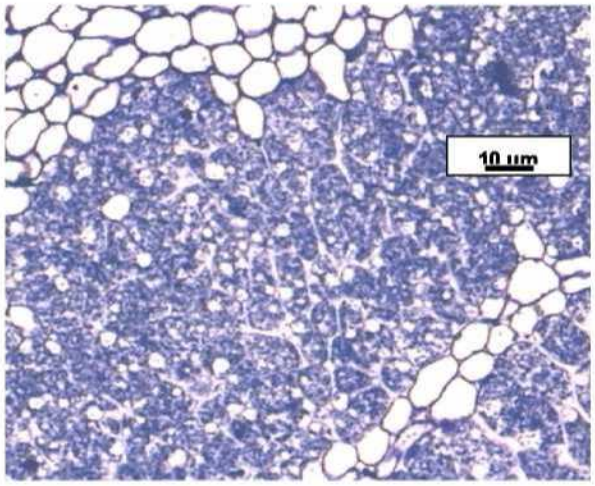

Figure 1. (A) : Partial view of the submerged part of stem in a *Pterocarpus officinalis* seedling. L, lenticels ; AR nod, adventive root nodules ; S nod, stem nodules ; R nod, root nodules. (B) to (E) : Longitudinal sections through stem nodules of *P. officinalis*. (B and C) : Serial thick sections (100µm) showing the direct link of the peripheral nodular vascular bundles (VB) to the stem (s) vascularization. (D) and (E) : thin sections through nodular stem nodules illustrating the numerous fixation zones (FZ) which cells are filled with densely packed bacteroids.

Table 1. Significance levels for growth and nutritionnal parameters in *Pterocarpus officinalis* seedlings at two water levels, and inoculated or not with *Bradyrhizobium* sp. and *Glomus intraradices* alone or together. S, significant at p<5% ; NS, not significant.

Trait	Water level	Inoculated status	Water level x Inoculated status
Height	S	NS	S
Collar diameter	S	S	S
Leave	S	S	NS
Stem	S	S	S
Root	NS	S	NS
Number of adventive roots	S	NS	NS
Adventive roots	S	NS	NS
Total biomass	S	S	NS
Number of root nodules	NS	S	S
Root nodules	NS	S	NS
Number of stem nodules	S	S	S
Stem nodules	S	S	S
Adventive root colonization	S	NS	NS
Root colonization	S	S	S
Nitrogen fixation in root nodules	NS	S	NS
Nitrogen fixation in stem nodules	S	S	S
N in leaves	NS	S	NS

P in leaves	NS	S	NS		

Table 2. Effect of inoculation with *Bradyrhizobium* sp. (*Br*) and *Glomus intraradices* (*Gi*) on growth of *Pterocarpus officinalis* seedlings under flooded conditions (P<5%).

Treatments	Height	Collar diameter	Leaf	Stem	Root
Number of adventive roots	Adventive roots (cm)	Total biomass (mg)	(mm)	(g)	(g)	(g)
Flooded					
Control	51.6 c	8.5 d	1.9 b	2,3 b	1.6 ab
4.1 b	20.1 b	5.8 bc			
Br	48,3 bc	7.1 bc	1.8 b	3.5 cd	1.4 ab
4.1 b	19.2 b	6.7 cd			
Gi	47.2 abc	8.3 d	1.9 b	3.2 c	1.9 b
4.6 b	21.3 b	7.1 d			
Br + *Gi*	53.8 c	8.0 cd	2.2 b	3.8 d	1.6 ab
4.8 b	23.4 b	7.6 d			
Non-flooded					
Control	40.6 a	6.1 b	1.0 a	1.5 a	1.1 a
0.0 a	0.0 a	3.6 a			

Br	47.5 abc	4.6 a	1.3 a	1.8 ab	1.5 ab
0.0 a	0.0 a	4.7 b			
Gi	51.5 c	4.9 a	1.7 b	2.1 b	1.8 ab
0.0 a	0.0 a	5.6 bc			
Br + *Gi*	42.8 ab	6.6 b	1.3 a	2.1 ab	1.5 ab
0.0 a	0.0 a	4.8 b			

Table 3. Effect of inoculation with *Bradyrhizobium* sp. (*Br*) and *Glomus intraradices* (*Gi*) on nodulation and mycorrhizal colonization of *Pterocarpus officinalis* seedlings under flooded conditions (P<5%).

Treatments	Number of root nodules	Root nodules (mg)	Number of stem nodules	Stem nodules (mg)
Adventive root colonization (%)	Root colonization (%)			
Flooded				
Control	5.3 a	22.0 a	2.3 b	0.4 a
0.0 a	0.0 a			
Br	44.6 bc	131.0 c	12.1 c	7.0 c
0.0 a	0.0 a			
Gi	3.3 a	41.0 ab	1.6 ab	0.4 a
56.2 b	66.1 d			
Br + *Gi*	60.3 c	162.0 c	10.5 c	4.0 b
65.8 b	69.6 d			
Non-flooded				

Control	1.3 a		8.0 a	0.0 a	0.0 a
-		0.0 a			
Br	50.5 c		181.0 c	0.0 a	0.0 a
-		0.0 a			
Gi	8.5 a		34.0 ab	0.0 a	0.0 a
-		28.6 b			
Br + *Gi*	31.8 b		112.0 bc	0.0 a	0.0 a
-		44.7 c			

Table 4. Effect of inoculation with *Bradyrhizobium* sp. (*Br*) and *Glomus intraradices* (*Gi*) on nitrogen fixation, N and P contents in leaves of *Pterocarpus officinalis* seedlings under flooded conditions (P<5%).

Treatments	Nitrogen-fixing root nodules (μmol C_2H_4/h/g nodule dry weight)		Nitrogen-fixing stem nodules (μmol C_2H_4/h/g nodule dry weight)
	N leaves (%)	P leaves (%)	
Flooded			
Control	1.75 a		0.00 a
	1.52 a	0.05 a	
Br	9.03 b		3.73 b
	2.10 b	0.06 ab	
Gi	1.83 a		0.00 a
	1.45 a	0.10 c	
Br + *Gi*	8.40 b		4.21 b
	2.16 b	0.11 c	
Non-flooded			

Control	0.00 a		-
	1.56 a	0.07 b	
Br	8.30 b		-
	2.21 b	0.06 ab	
Gi	1.88 a		-
	1.43 a	0.11 c	
Br + *Gi*	6.97 b		-
	2.23 b	0.13 c	

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Pattern of genetic diversity and gene flow of a tree species distributed in continental and insular Caribbean zones : case of *Pterocarpus officinalis* Jacq.

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Running title : pattern of diversity of a Caribbean tree

ABSTRACT

Aims : Patterns of genetic variation and gene flow are poorly documented for forest species in island ecosystems. The distribution of molecular variation for *Pterocarpus officinalis*, an tree species endemic to the Caribbean islands and south American continent, was analysed using microsatellite markers. The aims of this study were to quantify the genetic diversity, to assess the genetic structure and to analyse long distance gene flow.

Location : Leaves from 202 individuals of 9 *P. officinalis* populations dispersed on 5 different islands in the Caribbean and in one population of French Guyana in the South American continent were collected

Methods : We used chloroplast and nuclear microsatellite markers. Three universal chloroplast probes were selected to genotype 116 individuals. Six nuclear microsatellite loci were used to genotype 202 individuals. Classical models of populations genetics were used to analyse the data.

Results : Based on the combination of three universal chloroplast probes, ten individual chlorotypes were identified The nuclear microsatellite loci displayed a number of alleles ranging from 4 to 20. For both markers, the diversity parameters varied markedly among the populations, for chloroplast (H_{cp}=0.22 - H_{cp}=0.63) and nuclear markers (H_{emuc}=0.24 H_{emuc}=0.59). Island populations showed a lower value than continental populations (French Guyana), which was expected in the case of species distributed in small isolated islands. The fixation index ranged from F_{is} =-0.04 to F_{is} =0.37; except for Deshaies and Le Galion, a significant heterozygote deficit was detected. F_{st} was high for both chloroplast and nuclear microsatellites, F_{stcp}= 0.58 and F_{stnuc}= 0.29 respectively, resulting in a low pollen seed mediated ratio Rp/s= 2.18

Main conclusions : The significant F_{is} in some populations could result from selfing but it is likely to result from the crossing between relatives in those small and isolated populations. The distribution of chlorotypes, and the neighbour-joining tree based on both markers, presented a differentiation pattern that can be explained by the mode of seed dispersal by flotation in connection with the route of migration based on marine streams. Our results suggest that the populations from Caribbean islands partly derive from southern populations of Brazil and Guyanas.

Keywords : insularity, *Pterocarpus officinalis*, nuclear microsatellites, chloroplast microsatellites, genetic diversity, genetic structure, gene flow

Introduction

The understanding of interactions between gene flow, effective population size and genetic drift are fundamental to analysing the cause of genetic diversity within tree species. Although much research has been devoted to species distributed in continental and temperate zones (Hamrick *et al.,* 1992; Hamrick & Godt, 1996), very few studies have yet investigated this interaction in a tropical island ecosystem, which has numerous particularities compared to mainlands (Gómez *et al.,* 2003). Due to their isolated status, small size, and the small numbers of colonisers, islands exacerbate the effect of genetic drift, the effect of inbreeding depression and the differentiation between the populations within species (Barett, 1996). Numerous approaches in plants have been implemented to understand the pattern and process of species diversity and speciation using phylogenetic theory (Emerson, 2002). Experimental results in groups such as animals have confirmed the theoretical models and have indicated that differentiation between island populations is high and that islands contain less genetic variation (Frankham, 1996). However, the influence of many biophysical and biological factors in the pattern of within-plant genetic diversity in island systems is still poorly documented. For example, the interaction between the geographic distribution of (small) islands and the mode of seed dispersal and the distribution of genetic diversity is not well known. This is particularly true for tree species, although they represent an important component of the ecosystem and play a significant role in economic and environmental issues of islands.

Numerous tree species, however, undergo many threats due to overexploitation (Butaud *et al.,* 2005), reduction of the original forest ecosystem and the negative impact of invasive species (Tassin 1999). This is the case of *Pterocarpus officinalis* Jacq. (Leguminosae), one of the most important freshwater wetland tree species in the Caribbean (Bacon, 1990). It covers large areas of the coastal floodplain and individual trees and small stands occur along rivers and in the Luquillo Mountains (Cintrón, 1983). During the last two centuries, the distribution of *P. officinalis* was greatly reduced when coastal plain forests were cut and drained for agriculture. Today, most of the populations of *P. officinalis* are restricted to the extremes of their physiological distribution in areas adjacent to mangroves where changes in hydrology could affect the survivorship of these stands.

Several complementary approaches can be developed to investigate variation within tree species. Molecular markers with neutral effects clarify the impact of evolutionary forces such

as drift and migration, and provide basic information on the biology of the species. In this study we combined nuclear and chloroplast microsatellite markers.

Nuclear microsatellite markers are one of the most popular DNA-based approaches. They are co-dominant and among the less technically demanding and offer a fast method of providing information from variable loci, which is particularly efficient in species where genetic diversity is expected to be low. There are some limitations, however, owing to their size homoplasy (Navascués & Emerson, 2005; Estoup *et al.*, 2002).

Chloroplast microsatellite markers have received much attention and are frequently used in genetic diversity studies. They have been utilised for gymnosperm (Marshall *et al.* 2002; Gómez *et al.* 2003) and angiosperm tree species (Palme & Vendramin, 2002; Collevatti *et al.*, 2003; Grivet & Petit, 2003). Chloroplast microsatellites are maternally inherited in most angiosperms and disseminated by seeds, so the level of differentiation is greater than with bi-parental inheritance. They are then best suited to analyse gene flow by seeds.

Although molecular techniques have been widely used in tree species, few studies have combined both markers to study spatial genetic structure on different scales (Gamache *et al.*, 2003; Fontaine *et al.*, 2004; Andrianoelina *et al.*, 2005). This combination should provide useful information on colonisation and dispersal in tree species distributed in island and continental zones over both short and long periods (Petit *et al.*, 2005).

An analysis based on dominant AFLP markers of the differentiation between mainland and island populations of *P. officinalis* showed that island Caribbean populations differed from central America populations, but within these two regions it was difficult to determine relationships between populations (Rivera-Ocasio *et al.*, 2002). The authors assumed that these two sets could originate from the northern part of Brazil. After this first study, new research questions can be addressed: do we observed a lower diversity within population and a stronger differentiation among population, as expected in insular system and do we confirm the hypothetical route of migration from south America to Central America . Using six nuclear and three chloroplast microsatellite markers with a sample of nine populations dispersed over four different islands in the Caribbean and one in the northern part of the South American continent, the aims of our complementary study were then (i) to quantify the genetic variation within and between populations using both co-dominant nuclear and cytoplasmic markers, (ii) to compare the pollen- and seed-mediated gene flow in the insular system of the Caribbean, and, (iii) to analyse the geographic distribution of diversity in the natural range to infer the route of migration of the species.

METHODS

Species features

Pterocarpus officinalis (Fabaceae) grows throughout the Neotropics from 20°N to 2°S in latitude. *P. officinalis* is one of the dominant tree species in fresh and brackish water wetlands throughout the Caribbean, and coastal areas of central and northern South America (imbert *et al*., 2000). The tree can reach 25 m tall with a diameter at breast height around 0.5 metres. The species grows in monospecific stands with adult tree densities reaching 200 individual trees ha^{-1}. It is characterized by a narrow buttress, compound and alternate leaves, yellow bee-pollinated flowers and bloodred sap (Rivera-Ocasio *et al.*, 2002; Little & Wadsworth, 1964). Seeds are disseminated by flotation and are still able to germinate after several weeks in sea water (Lopez, 2001; Rivera-Ocasio *et al*., 2002).

Plant material

Leaves from 202 individuals of 9 populations dispersed over five different islands in the Caribbean and one on the South American continent were collected between March 2003 and September 2003. On each island, populations were defined as the set of individuals exchanging genes without any geographical disruption. The number of populations varied from one island to another. Populations were generally small and scattered on each island, except for Belle Plaine, which has numerous individual trees in a large area.

Sampling was, however, very unbalanced, the number of individuals per island varying from 13 (Puerto Rico) to 33 (Belle Plaine) (Table 1). The objective was to sample a minimum of 20 individuals per population. Five leaves were collected from each tree and immediately dried using silica gel. Where the size of the population was sufficient, we selected trees separated by more than 10 m to avoid selection of the same clone (assuming that resprouting by root suckers can create patches of the same clone).

DNA extraction

DNA was extracted from dried leaves following the modified protocol described by Bousquet *et al.* (1990). Leaves (100 mg) were ground to a fine powder with a mortar and pestle under

liquid nitrogen. DNA-extraction buffer (5 mL) was added (100 mM Tris–HCl pH 8.0; 1.4 M NaCl; 20 mM ethylenediaminetetraacetic acid (EDTA); 2% mixed alkyl trimethyl ammonium bromide (MATAB); 1% polyethylene glycol (PEG) 6000) and the mixture was transferred to 13-mL sterile tubes.

The tubes were then incubated at 65°C for 30 min. Samples were washed with wet chloroform (chloroform: isoamyl alcohol, 24: 1) to remove cellular debris and protein. After 15-min centrifugation at 4500 g, the liquid phase was transferred to new 13-mL tubes. Samples were again washed and centrifuged and the liquid phase was transferred to new 13-mL tubes. Sodium acetate (pH 5, 3 M, 450 µL) and isopropanol (5 mL) were added and mixed gently before putting the sample in the deep freeze for 15 min 20 °C) to precipitate the DNA. Then, after 5 min centrifugation at 4500 g, the isopropanol was eliminated. The resulting DNA pellets were washed with 70% ethanol (1 mL), transferred to 1.5-mL Eppendorf tubes, and centrifuged for 5 min at 1900 g. After elimination of the ethanol, the pellet was dried and resuspended in 200 µL sterile water for 20 min at 37 °C. Samples were stored at -20 °C until required.

Chloroplast microsatellite method

Seven universal microsatellite primers (Ccmp) described by Weising & Gardner (1999), and 33 solanaceous microsatellites (Ntcp) described by Bryan *et al.* (1999), were tested over a subset of the population. Among the 40 primer pairs tested on a sample of eight individuals, three were polymorphic (Ccmp3, Ccmp7 and Ntcp8). For the Ccmp primers, PCR amplifications were carried out in a 10 µL reaction mix, with 2 µL DNA (1 ng/µL), 5 µL 2·X buffer, 0.5 µM of each primer (R and F), and 0.1 U/µL polymerase DNA Taq, completed with sterile water. All reactions were performed in a 6 Stratagene Thermocycler. Optimal amplification conditions were 1 cycle of 5 min at 94 °C (initial denaturation), followed by 30 cycles of 1 min at 94 °C (denaturation), 1 min at 56 °C (annealing), and 1 min at 72 °C (extension) and a final step of 8 min at 72 °C ensured full extension of all amplified products. For the Ntcp primers, PCR amplifications were carried out in a 10 µL reaction mix with 2 µL DNA (1 ng/µL), 5 µL 2X·buffer, 0.2 µM of each primer (R and F), and 0.025 U/µL polymerase DNA Taq, completed with sterile water. All reactions were performed in a Stratagene Thermocycler. Optimal amplification conditions were 1 cycle of 5 min at 94 °C,

followed by 30 cycles of 45 s at 92 °C, 45 s at 55 °C, and 1 min at 72 °C, and a final step of 8 min at 72 °C. Bands were separated and visualized in acrylamide gel.

Nuclear microsatellite method

The genetic analysis was done using six nuclear microsatellites: *mPoCIRE01, mPoCIRH08, mPoCIRE09, mPoCIRF03, mPoCIRF08, and mPoCIRE04,* designed specifically for *Pterocarpus officinalis Jacq.* Their characteristics and the methods used to obtain them are described elsewhere (Muller *et al.,* in press).

Data analysis

Nuclear microsatellites studied as allele frequencies, the number of alleles per locus (na_{nuc}), observed heterozygosity (H_{Onuc}) and expected heterozygosity (He_{nuc}) (Nei, 1978), and the fixation index (F_{is}), per population were computed using Genetix 4.03 (Belkhir *et al.*, 2001).

To check if diversity estimates were affected by the differences in sample sizes and the various spatial scales over which individuals were pooled into 'populations', we calculated the allelic richness per population and island taking into account the dependence on sample size with an adaptation of the rarefaction index of Hurlbert (1971) (El Mousadik & Petit, 1996), named '*R*', using Fstat 2.9.3.2 (Goudet, 2001). The principle is to estimate the expected number of alleles in a sub-sample of 2 *n* genes, given that 2 *N* genes have been sampled ($N > n$). In Fstat, *n* is fixed as the smallest number of individuals typed for a locus in a sample. The pairwise F_{stnuc} was calculated among the 9 populations by Fstat (Goudet *et al.*, 2002). Testing for population differentiation was conducted by bootstrapping, and probability values were determined per 1000 permutations according to the approach described by Excoffier *et al.* (1992) and compared to the P value adjusted by the sequential Bonferroni procedure (Rice, 1989).

To explain the departure from Hardy-Weinberg equilibrium within population and to detect the number of sub-populations within a group of genotypes, we used the method developed by Guillot *et al.* (2005a) and the Geneland package (Guillot *et al.,* 2005b). This method is based on a bayesian model implemented in a Markov chain Monte Carlo scheme.

A chlorotype is defined as a combination of the different alleles established at each locus. Because of the non-recombining nature of the chloroplast genome, cpDNA chlorotypes were then treated as alleles at a single locus. Chlorotype diversity and genetic structure parameters were calculated using Arlequin software version 2000 (Schneider *et al.*, 2000). The gene diversity index H_{cp} was calculated using the Nei formula (Nei, 1987). The number of chlorotypes (na_{cp}) and the effective chlorotype number (ne_{cp}) were calculated for each population. The genetic structure was estimated using analysis of molecular variance, AMOVA (Excoffier *et al.*, 1992). The pairwise F_{stcp} was calculated among the 9 populations. The test for population differentiation was conducted as for nuclear markers.
The minimum spanning network between haplotypes was computed with Minspnet (Excoffier & Smouse 1994), and with Arlequin 2000 (Schneider *et al.*, 2000).

For both markers (nuclear and chloroplast) the genetic structure illustrated by a cluster analysis using the neighbour-joining method and Phylip 6.1 software (Felsenstein, 1993). The matrix of genetic distances was calculated using the Cavalli-Sforza distance D2 (Cavalli-Sforza & Edwards, 1967), which is adapted to describe the structure of populations according to their evolutionary history. The spatial pattern of distribution of genetic diversity was studied by means of a Mantel test which was used to estimate the correlation between the matrix of geographical distances between populations and that of the genetic distances estimated by pairwise F_{st}.
Gene flow by seed and pollen was compared following Ennos (1994) and (Tarayre *et al.*, 1997). We estimated the pollen/seed flow ratio ($R_{p/s}$) using the Ennos (1994) formula considering the extent of inbreeding within populations (F_{is}): $R_{p/s} = [A(1+F_{is})-2C]/C$, where $A=(1/F_{stnuc} - 1)$ and $C=(1/F_{stcp} - 1)$.

RESULTS

Within-population diversity

The six nuclear microsatellite loci were all polymorphic and the number of alleles per locus ranged from 4 for *mPoCIRE01* to 20 for *mPoCIRF08*. Mean number of alleles per locus per population ranged from na_{nuc}=1.67 in the El Yunque location on the Puerto Rico island (allelic richness in this population R=1.64) to na_{nuc}=5.33 in la Crique Alexandre Jacques in

French Guyana (R=4.12). Observed and expected (in parenthesis) heterozygosity values ranged from Ho_{nuc}=0.20 (He_{nuc}=0.26) in El Yunque to Ho_{nuc}=0.50 (He_{nuc}=0.59) in la Crique Alexandre Jacques. The ranking of population for na_{nuc} and Ho_{nuc} was similar but differed with the allelic richness R. The fixation index F_{is} varied among populations, ranging from F_{is} =-0.04 in Le Galion to F_{is} =0.37 in the Indian River site on the Dominica Island. Except for Deshaies and Le Galion, a significant heterozygote deficit was detected in each population (Table 2).

Among the 202 individuals genotyped with chloroplast microsatellites, 116 provided a chlorotype with three primers, and the other individuals were eliminated due to the absence of amplification for one of the three primers. Using two primers, the number of individuals presenting a chlorotype was higher, but we chose to conduct the analysis with chlorotypes defined by three primers to get a better accuracy of the genetic relationship between populations and to reduce the effect of homoplasy (Navascues & Emerson, 2005).

Based on the combination of these three universal chloroplast probes, ten individual chlorotypes could be identified (Table 3). Four of these chlorotypes (A, C, D and F) were more widespread than the six others. The frequencies of the four widespread chlorotypes ranged between 0.25 and 0.16, whereas the frequency of the remaining chlorotypes varied between 0.03 and 0.09 (Table 2). The two more prevalent chlorotypes, F and D, were found in 5 and 6 of the 9 studied populations, respectively.

The diversity parameters varied markedly among the populations (Table 2), the least variable population being Le Moule in Guadeloupe island (H_{cp}=0.22) and the most variable being Indian River in the Dominican Republic (H_{cp}=0.68). A similar pattern was observed for na_{cp} but not ne_{cp}, this parameter being affected by the distribution of chlorotype frequency. The variation among populations was correlated with sample size, the least variable populations having the smallest number of individuals.

Differentiation between populations

Values of F_{stnuc} calculated with nuclear microsatellites showed a marked differentiation between populations (F_{stnuc}=0.29, P<0.0001) (Table 4). When estimated with just the three populations located in the two main and contiguous islands of Guadeloupe (Basse Terre and Grande Terre, Fig.1), F_{stnuc} dropped but remained high (F_{stnuc}=0.16, P<0.001), suggesting a

low gene flow between the three populations within this island. This differentiation was illustrated by the neighbour-joining tree (Fig 2a) which showed bootstrap values higher than 500 and clearly separated the cluster of the Guadeloupe archipelago and the cluster of the Puerto Rico populations. Surprisingly, the population of French Guyana was located between these two clusters although it is geographically very distant. The scattered points between the genetic and geographic distances did not present a linear relationship (Fig. 3a) and the associated Mantel test confirmed this observation, confirming a non-significant coefficient of correlation (r=-0.213, P=0.668).

The differentiation index assessed with the chloroplast microsatellites including all the populations exhibited a high value too (F_{stcp} = 0.58, P<0.0001), but remained high when the parameter was estimated within Guadeloupe island (F_{stcp}=0.45, P<0.0001). The neighbour-joining tree showed a more complex pattern than for the nuclear microsatellites (Fig. 2b); the populations from the Guadeloupe archipelago were distributed in two clusters, one close to Puerto Rico populations, the other one close to Dominica. The French Guyana population was still found between the cluster of Guadeloupe and the cluster comprising Martinique, Dominica, Marie Galante and Puerto Rico. Figure 3b does not illustrate a linear relationship between genetic and geographic distances, like the nuclear microsatellites. This absence of linear correlation was confirmed by the Mantel test which indicated a non-significant correlation between genetic and geographic distances (r=0.026, P=0.26).

The distribution of the 10 haplotypes across the natural range showed a complex pattern (Figure 4a). Haplotypes C, D and H were scattered among distant populations (C, D and H were present in French Guyana, and in the islands from Martinique up to Puerto Rico), whereas some others such as haplotypes E and I were restricted to a single population. Haplotype relatedness represented by the minimum spanning tree (Fig. 4b) did not exhibit frequent haplotypes occupying a central position. On the one hand, some frequent and rare haplotypes were closely connected in the tree and present in the same population, for example A and E in Le Moule in Guadeloupe or F and I in Marie Galante island. On the other hand, some distant haplotypes in the minimum spanning tree were found in the same population, for example C and F in Deshaies in Guadeloupe.

The pollen/seed flow ratio estimated according to Ennos (1994) for the total population was Rp/s= 2.18. To compare the patterns of gene flow over long and short distances, a similar approach was applied to the 3 populations of Guadeloupe island. Although the populations were very closed, the ratio presented a similar value Rp/s= 2.88.

DISCUSSION

Within-population diversity

The expected heterozygosity parameters assessed with nuclear microsatellites in this study were among the smallest compared to other studies using the same marker (Table 5). Our estimates are close to values of species distributed in small islands such as *Santalum austrocaledonicum* and *Santalum insulare* (Table 5).

The results obtained with *P. officinalis* confirm some theoretical models related to the pattern of diversity in islands. Expected heterozygosity shows a higher value in the population of French Guyana (He_{nuc}=0.59), the highest *He* for an island population being the population of Belle Plaine (He_{nuc}=0.49). This difference was more pronounced when the allelic richness R_{nuc}, which takes into account the sample size, was used to make the comparison (R_{nuc} =4.12 in French Guyana and R_{nuc}=3.02 in Belle Plaine). Although the differences are smaller than the standard deviation, they suggest that within-population diversity is higher in the mainland population than in the island population. These results confirm the first analysis on *P. officinalis* using AFLP: this study showed that the diversity was lower in the islands (Puerto Rico, Dominican Republic, Guadeloupe and Trinidad) than in four populations of the continent (Venezuela, Costa Rica, Panama and Darien region) (Rivera-Ocasio *et al.,* 2002). They also confirm previous general results obtained by comparing the diversity parameters of various organisms for island and mainland populations (Frankham, 1996). Oceanic islands are expected to lose genetic variation at foundation and after foundation as these populations have lower population sizes than mainland populations. In addition, populations in most of the Caribbean islands have undergone marked reduction due to human activities thereby contributing to the reduction of diversity (Rivera-Ocasio *et al.,* 2002; Imbert *et al*., 1988).

Fixation index

Except for two populations, our study reveals a strong heterozygote deficit with significant F_{is} values ranging from F_{is}=0.17 (P<0.0001) to F_{is}=0.37 (P<0.0001). This result is similar to that found in other forest tree species present in small islands (Bottin *et al*., 2005). There are various possible explanations for this deficit.

The first is the presence of null alleles which can be addressed by the analysis of missing data. The percentage of missing data was especially high for some loci, suggesting that null alleles may be present (the percentage of missing data per locus is as follows mPoCIRE01: 4.5%; mPoCIRH08: 5.1%; mPoCIRE09: 3.2%; mPoCIRF03: 8.3%; mPoCIRF08: 12.1%; mPoCIRE04:14%). However the estimation of F_{is} using loci with a very low percentage of missing data (mPoCIRE01, mPoCIRH08 and mPoCIRE09) gave very similar results. Additionally, these three suspicious loci (mPoCIRF03, mPoCIRF08 and mPoCIRE04) did not give systematically positive and very high values of F_{is} for each locus, suggesting that the missing data do not result from a null allele effect.

The second explanation is the Whalund effect which occurs when a spatial or temporal structure exists in the sampled population. The presence of the Whalund effect may not be the most relevant explanation because the size of each population was very small in each location and no sub-population included in the total population was noticed during sampling. In addition, the sampled individual trees belong to the same adult cohort and no temporal sub-structure was observed during the collection of leaves. This observation was confirmed by the result of the of the method developed by Guillot *et al.* (2005a and b) to detect sub-structure. No sub-populations were detected within each population showing a positive F_{is} .

The third explanation is the mating system of trees; selfing can increase the rate of homozygosity in the population. Selfing is favoured by a low density of trees within a population and a lack of pollinators. Tree density was generally high in most of the populations, thus facilitating the exchange of pollen, and does not seem to be the most relevant explanation. However, in some populations such as Belle Plaine in Guadeloupe, abundant flowering within the same tree was observed, which will facilitate self-pollination.

The fourth explanation is that the high positive F_{is} results from the finite population size. The small number of individuals at foundation and the small population size facilitate crossing between relatives and increase inbreeding. This theoretical principle was empirically confirmed by Frankham (1998), who found higher values of F_{is} in endemic island species compared to the mainland. Our results show, however, that the population in French Guyana presents a positive and significant F_{is} which is in contradiction with Frankham's analysis. This is explained by the isolated status of this population in French Guyana

Patterns of differentiation and pollen seed-mediated gene flow

Because *P. officinalis* is distributed in a fragmented range, the F_{stnuc} estimated using nuclear microsatellites is expected to be higher than in species distributed in continuous range. This result was confirmed by the comparison with F_{stnuc} assessed for continental tree species with a continuous range (Table 5). Table 5 shows smaller differentiation indices, but similar F_{stnuc} are observed in other insular species such as *Santalum austrocaledonicum* and *S. insulare*. This pattern is not verified when the differentiation is assessed using chloroplast microsatellites. *P. officinalis* does not give F_{stcp} estimates higher than those found in continental species with a continuous range (Table 5). This result can be explained by the mode of seed dispersal of the latter species, i.e. gravity or animals with limited territory leading to the strong structure. Generally, the differentiation parameter is higher with cytoplasmic markers than nuclear markers (Petit *et al.,* 2005), even in insular systems (Bottin *et al.,* 2005; Bouvet unpublished data), this general result was confirmed in our study. Our analysis presents new results that enable comparison of seed and pollen flow in a species distributed in both continental zones and small islands. The few studies combining nuclear and cytoplasmic markers are not abundant in angiosperm tree species (Petit *et al.*, 2005), they have shown that pollen to seed flow ratios are generally high for some angiosperm tree species that are wind-pollinated and distributed over a continental range and for gymnosperm species. For example, this is the case for temperate species such as *Fagus silvatica* ($R_{p/s}$=84), *Quercus robur* ($R_{p/s}$=286), *Quercus petraea* ($R_{p/s}$=500) (King and Ferris 1998) and for *Picea mariana* ($R_{p/s}$ =77) (Gamache *et al.,* 2003). Ratios are smaller for species whose pollen is disseminated by insects and seeds by gravity or animals: *Vitellaria paradoxa* ($R_{p/s}$ =47) (Fontaine *et al.,* 2004) and for *Dalbergia monticola* ($R_{p/s}$ =15) (Andrianoelina *et al.,* 2005). This ratio is different in *P. officinalis* ($R_{p/s}$= 2.18) because this species is distributed in fragmented range, it is insect pollinated and seeds are mainly dispersed through flotation. This value is close to the estimates obtained with two other insular tree species *Santalum austrocaledonicum* in New Caledonia ($R_{p/s}$=3.82) and *Santalum insulare* in French Polynesia ($R_{p/s}$=2.13) whose seeds are dispersed by birds and flowers are insect pollinated (Bouvet unpublished data).

Origin of Caribbean populations

In a previous study of *P. officinalis* based on AFLP, Rivera-Ocasio *et al.* (2002) analysed the differentiation between mainland populations distributed in central and northern South

America and island populations. They showed two clusters separating continental populations (Venezuela, Panama and Costa Rica) from island population (Guadeloupe, Dominican Republic, Trinidad and Puerto Rico). The authors proposed some possible scenarios of colonisation in the new tropics. Based on pollen records, *P. officinalis* is a relatively recent colonizer of the Neotropics (Graham, 1995) and is considered to derive from the West African species *P officinalis giletii*. In one of their scenarios, Rivera-Ocasio *et al.* (2002) suggested that populations from America could be colonised by seeds transported by the Benguela current, which originates off the south-western coast of Africa and makes contact with the north-eastern coast of Brazil. They suggest sampling in the eastern extreme distribution e.g. northern Brazil and the Guyanas and from African populations, to clarify the colonization history of the species.

In our study, the use of pollen and seed dispersal markers and new sampling in the region allow for a complementary approach to analysis of the phylogeographic pattern of *P. officinalis*. With nuclear and chloroplast microsatellites, the Mantel test does not indicate a pattern of isolation by distance, showing that geographically close populations are genetically close. This pattern is also illustrated by the neighbour-joining trees showing that the continental population from French Guyana is not separated from the island populations. With nuclear markers, the population of French Guyana occupies a central position between the clusters of Puerto Rico and the Guadeloupe archipelago (Fig. 2a). With chloroplasts we observe a similar structure but with an additional complexity due to the position of Marie Galante outside the Guadeloupe cluster (Fig. 2b). Although homoplasy may be the cause of numerous identities per state, with highly variable stepwise mutating markers such as microsatellites (Estoup *et al.*, 2002; Nesvacués & Emerson, 2005), this differentiation pattern can be explained by the mode of dispersal of seeds by flotation in connection with the route of migration based on marine streams suggested by Rivera-Ocasio *et al.* (2002) (Fig. 1). Our chloroplast microsatellites show that seeds from the north-eastern coast of South America can be dispersed through the Caribbean islands by marine streams. Our results are consistent with the assumption of Rivera-Ocasio *et al.* (2002). Additionally, according to this first study showing marked differences between continental and island populations, and then a very limited gene flow between these two regions, our results suggest that the population from Caribbean islands derive mainly from southern populations of Brazil and Guyanas. However, new sampling and analyses are needed to confirm this seed flow along the American coast and the Caribbean and the origin of West Africa.

Conclusion

P. officinalis is distributed over a large area in the American continent and in small islands of the Caribbean Sea. Although the species seems to be abundant in the continental zone, the size of the remaining populations in the islands is critical. Some populations in Dominica and in Guadeloupe comprised only a few trees. In addition, the high fixation index found in some island populations is likely to indicate that inbreeding is present in some populations. Extinction due to inbreeding is among the most serious threats in small islands (Frankham, 1998). Urgent measures need to be taken in some islands to protect *P. officinalis* and to implement a strategy of conservation.

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Biosketches

Félix Muller is studying for a PhD at the University of Antilles-Guyane in Guadeloupe in collaboration with CIRAD, where he is analysing the pattern of diversity of *Pterocarpus officinalis* and the symbionts associated with the tree. He is also conducting experiments to clarify the role of symbiosis in the adaptation of the species in flooded zones.

Marie Voccia is an MSc student in forestry in the forestry school of ENGREF in Nancy, France.

Amadou Bâ is a professor at the University of Antilles-Guyane involved in research related to the functioning of symbiosis in various ecological contexts.

Jean-Marc Bouvet is the head of the research unit 'Genetic diversity and breeding of forest tree species' in the Forestry Department of CIRAD. His research interest is the analysis of the impact of evolutionary forces on genetic diversity using molecular tools and quantitative data in forest tree species.

Fig. 1: Main occurrence of *Pterocarpus officinalis* in the region and location of the populations analysed in the study.
The directions of the main marine currents are represented by arrows (according to Rivera-Ocasio (2002) and http://bulletin.mercator-).

Fig. 2: Unrooted neighbour-joining tree of (a) nuclear microsatellites and (b) chloroplast microsatellites drawn with Phylip 6.1 (Felsenstein, 1993) with the matrix of genetic distances calculated using the Cavalli-Sforza distance (Cavalli-Sforza & Edwards, 1967). Bootstrap values are presented at the base of branches.

Fig. 3: Relationship between genetic and geographical distances among populations of *Pterocarpus officinalis* Jacq.. Matrices of genetic distances were calculated using pairwise (a) nuclear *Fst* and (b) chloroplast *Fst* (Arlequin ver. 2000).

Fig. 4 (a) Chlorotype distribution of *Pterocarpus officinalis* jacq. in the Caribbean. The size of the circle is proportional to the size of population sampled.

Fig. 4 (b) : Minimum spanning network among the 17 chlorotypes found in the natural range of *Pterocarpus officinalis* Jacq.. Circle size is proportional to the frequency of each chlorotype (identified by a letter) in the total population.

Table 1: Co-ordinates, sample size (N) and main characteristics of the populations of *Pterocarpus officinalis*

Country	Island name	Population name	Lat. (N)	Long.(W)	N	Characteristics
Guadeloupe (France)	Grande terre	Belle Plaine	16°20'	61°32'	36	Several hundred trees in a monospecific stand of 5 ha
	Basse terre	Deshaies	16°18'	61°48	20	very small population (20 residual trees)
	Grande Terre	Le moule	16°20'	61°21'	20	very small population (50 dispersed trees)
	Marie Galante	Bois de Folle-Anse	15°56'	61°18'	20	edge of river (several hundred trees)
Dominican Republic	Dominica	Indian River	15°34'	61°28'	20	edge of river (30 residual trees)
Martinique (France)	Martinique	le Galion	14°45'	60°55'	20	last Martinique population (one hundred residual trees)
Puerto Rico	Puerto Rico	El Yunque	18°19'	65°47'	13	Mountain population altitude 400 m
	Puerto Rico	Sabana Seca	18°28'	66°22'	20	Coastal population of 12 ha
French Guyana (France)	-	la Crique Alexandre	5°24'	52°58'	33	small population along the affluent of the Sinnamary river

Table 2: Diversity parameter assessed with nuclear and chloroplast microsatellite markers in the population of *P. officinalis*.
N number of individuals; na, number of alleles per locus; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *Fis*, fixation index; *R*, corrected allelic richness. *P* values: ns; $P > 0.05$*; $P < 0.05$ **; $P < 0.01$***; $P < 0.001$ (*P* values were adjusted using the sequential Bonferroni procedure, Rice (1989))

Island / Country	Population	Nuclear Microsatellites						Chloroplast Microsatellites			
		N_{nuc}	na_{nuc}	R_{nuc}	Ho_{nuc}	He_{nuc} (SD)	F_{is}	N_{cp}	na_{cp}	ne_{cp}	H_{cp} (SD)
Guadeloupe	Belle Plaine	36	4.50	3.02	0.44 (0.23)	0.49 (0.28)	0.11**	28	4	2.56	0.63 (0.06)
Guadeloupe	Deshaies	20	2.50	2.20	0.38 (0.32)	0.38 (0.31)	0.03 ns	15	3	2.06	0.36 (0.14)
Guadeloupe	Le moule	20	2.83	2.63	0.36 (0.20)	0.45 (0.24)	0.25**	9	2	1.25	0.22 (0.17)
Marie Galante	Bois de Folle Anse	20	2.50	2.00	0.36 (0.20)	0.24 (0.21)	0.22**	16	3	2.25	0.36 (0.07)
Dominica	Indian River	20	3.33	2.58	0.25 (0.22)	0.38 (0.23)	0.37***	18	3	2.79	0.68 (0.06)
Martinique	le Galion	20	4.17	3.02	0.42 (0.34)	0.39 (0.27)	-0.04 ns	10	3	1.52	0.38 (0.18)
Puerto Rico	El Yunque	13	1.67	1.64	0.20 (0.28)	0.26 (0.24)	0.31**	-	-	-	-
Puerto Rico	Sabana Seca	20	3.17	2.58	0.47 (0.19)	0.46 (0.12)	0.04*	12	2	1.95	0.53 (0.08)
French Guyana	la crique Alexandre	33	5.33	4.12	0.50 (0.22)	0.59 (0.24)	0.17***	8	3	1.68	0.46 (0.20)
Total		202	9.67	-	0.36 (0.17)	0.57 (0.21)	0.36***	116	10	3.67	0.63 (0.07)

Table 3: Frequencies of chlorotypes in the total population of *Pterocarpus officinalis* Jacq., allelic characteristics in base pairs for the three loci, and allelic combination corresponding to each chlorotype

Chlorotype	Chlorotype frequency	allele description in number of base pair		
	%	CCMP3	CCMP7	NTCP8
A	16,38	95	154	244
B	2,59	97	156	244
C	23,28	97	156	248
D	20,69	96	154	245
E	0,86	95	153	244
F	25,00	96	153	245
G	0,86	96	153	244
H	8,62	96	155	245
I	0,86	96	152	245
J	0,86	97	156	245

Table 4: Analysis of molecular variance showing the percentage variation and the Fst with nuclear and chloroplast (between brackets) microsatellites using all the populations and just populations of the Guadeloupe island

	All populations			Populations of Belle Plaine-Le Moule-Deshaies		
Source of variation	d.f.	Percentage variation	F_{struc} (F_{stcp})	d.f.	Percentage variation	F_{struc} (F_{stcp})
Among populations	8 (7)	29.19 (58.42)	0.29 (0.58)	2 (2)	15.87 (45.19)	0.16 (0.45)
Within populations	305 (108)	70.81 (41.58)		121 (49)	84.13 (54.81)	
Total	313 (115)			123 (51)		

Table 5: Main parameters of diversity and differentiation between populations estimated with nuclear and chloroplast microsatellites in some tree species. Comparison with the present study
n: sample size, *He*: expected heterozygosity, *Fst*: differentiation index

Species	species characteristics		nuclear studies				chloroplast studies			
Species	distribution	mode of dispersal	n	H_{enuc}	F_{stnuc}	Reference	n	H_{cp}	F_{stcp}	Reference
Pterocapus officinalis jacq.	insular	barochore/hydrochore	202	0.24-0.59	0.29	present study	116	0.22-0.68	0.58	this article
Santalum insulare	insular	zoochore	162	0.27-0.56	0.23	Bouvet (unpublished)	343	0.40 -0.67	0.67	Butaud et al.(2005)
Santalum austrocaledonicum	insular	barochore/zoochore	431	0.11-0.74	0.33	Bottin et al. (2005)	218	0.00-0.69	0.60	Bottin *et al.* (np)
Caryocar brasiliense	continental	barochore/zoochore	314	0.58–0.85	0.11	Collevatti et al. (2001)	160	-	0.84	Collevatti et al. (2003)
Dalbergia monticola	continental	barochore/zoochore	-	-	-	-	100	0.00-0.80	0.57	Andrianoelina et al. (2005)
Vitellaria paradoxa	continental	barochore/zoochore	169	0.25–0.42	0.05	Sanou et al. (2005)	116	0.00-0.49	0.88	Fontaine et al. (2004)
Corylus avellana	continental	barochore/zoochore	-	-	-	-	248	0.43	0.85	Palme & Vendramin (2002)
Grevillea macleayana	continental	barochore	130	0.42–0.53	0.22	England et al. (2002)				
Vouacapoua americana	continental	zoochore	408	0.34–0.52	0.08	Dutech et al. (2004)				
Symphonia globulifera	continental	barochore/zoochore	914	0.72-0.85	-	Aldrich et al. (1998)				
Swietenia macrophylla	continental	barochore/zoochore	194	0.78-0.81	0.10	Lemes et al. (2003)				
Swietenia macrophylla	continental	barochore/zoochore	284	0.59-0.80	0.11	Novick et al. (2003)				
Melaleuca alternifolia	continental	barochore/zoochore	500	0.13–0.92	0.07	Rossetto et al. (1999)				

Fig. 1:

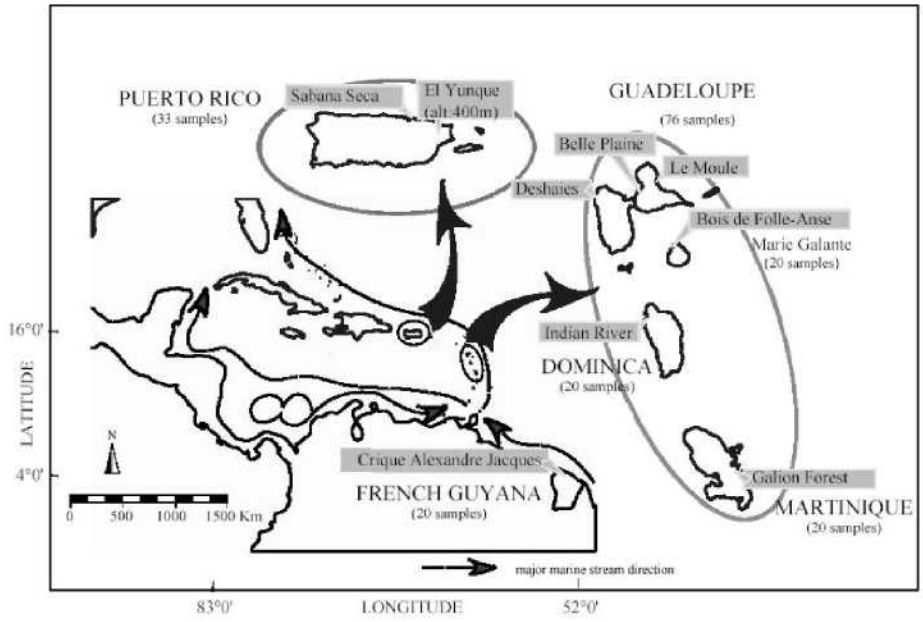

PUERTO RICO
(33 samples)
Sabana Seca
El Yunque
(alt:400m)
GUADELOUPE
(76 samples)
Belle Plaine
Le Moule
Deshaies
Bois de Folle-Anse
Marie Galante
(20 samples)
Indian River
DOMINICA
(20 samples)
Crique Alexandre Jacques
FRENCH GUYANA
(20 samples)
Galion Forest
MARTINIQUE
(20 samples)
N
0 500 1000 1500 Km
major marine stream direction
16°0'
4°0'
LATITUDE
83°0'
LONGITUDE
52°0'

Fig. 2 (a)

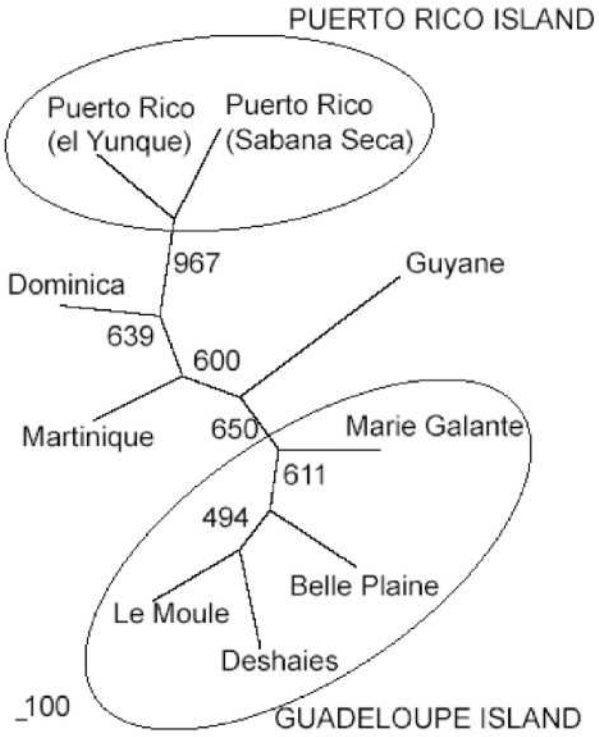
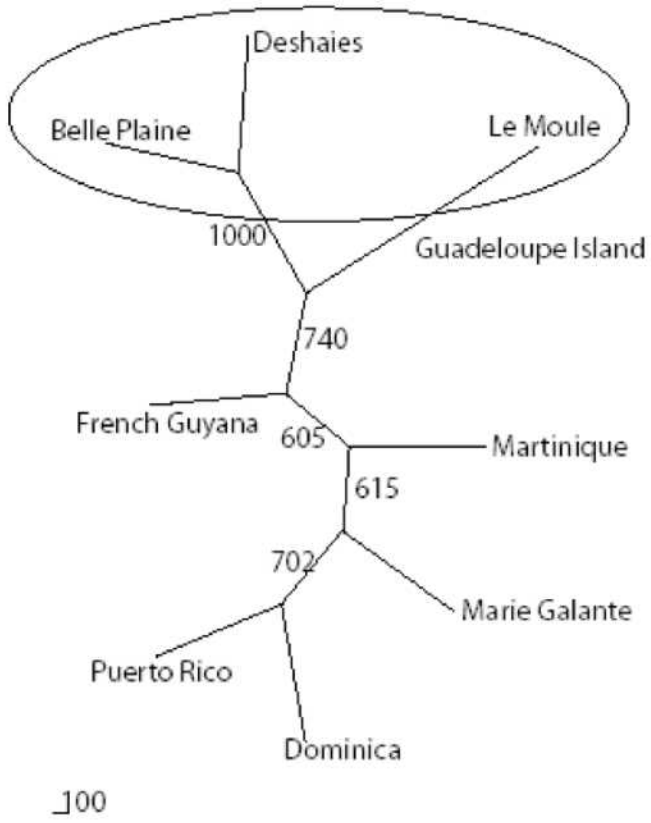

PUERTO RICO ISLAND
Puerto Rico (el Yunque)
Puerto Rico (Sabana Seca)
967
Dominica
Guyane
639
600
Martinique
650
Marie Galante
611
494
Belle Plaine
Le Moule
Deshaies
100
GUADELOUPE ISLAND

Fig. 2 (b)


Deshaies
Belle Plaine
Le Moule
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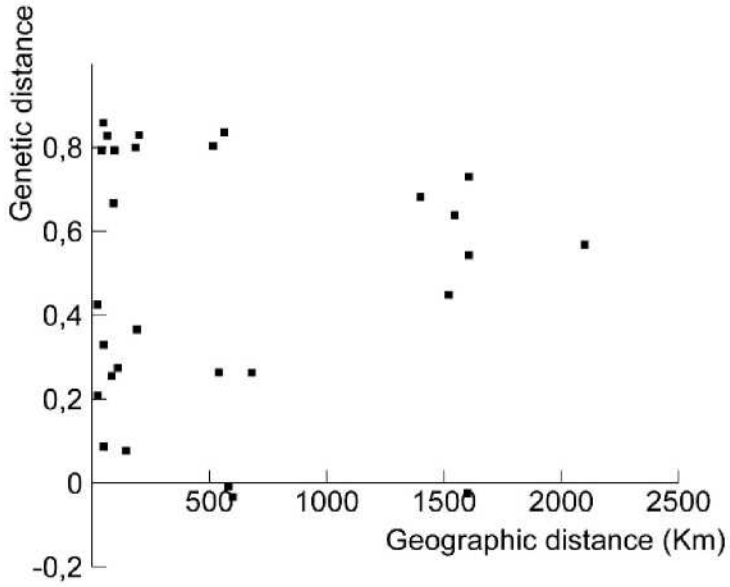
Guadeloupe Island
740
French Guyana
605
Martinique
615
702
Marie Galante
Puerto Rico
Dominica
100

Fig. 3: (a)


Genetic distance
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0,2
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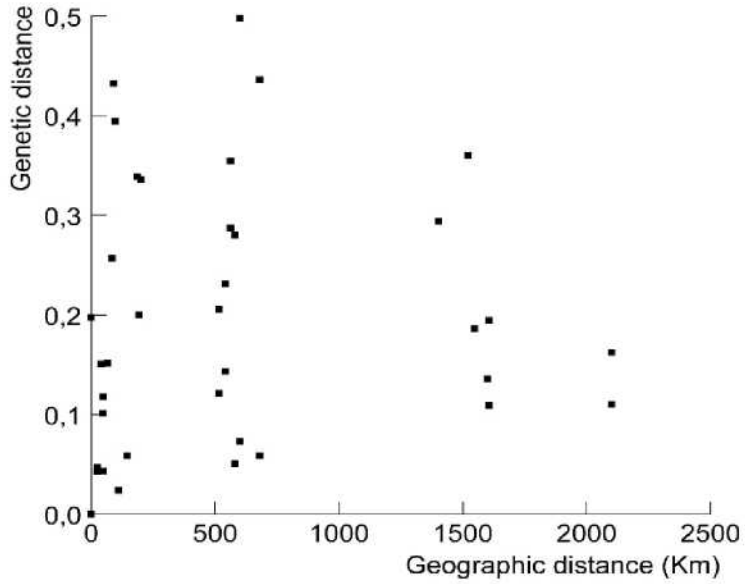
500
1000
1500
2000
2500
Geographic distance (Km)

Fig. 3: (b)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1500
2000
2500
Geographic distance (Km)

Fig. 4: (a)

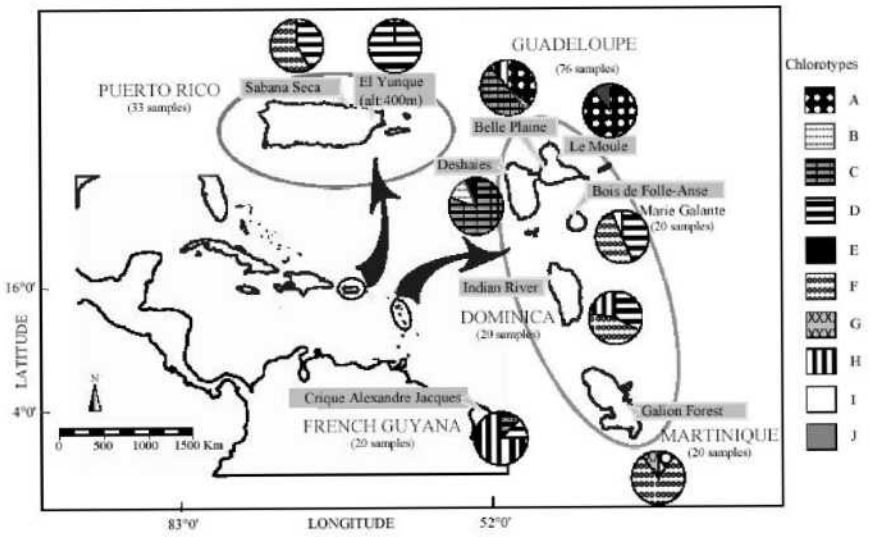
PUERTO RICO
(33 samples)
Sabana Seca
El Yunque
(alt 400m)
GUADELOUPE
(76 samples)
Belle Plaine
Le Moule
Deshaies
Bois de Folle-Anse
Marie Galante
(20 samples)
Indian River
DOMINICA
(20 samples)
Crique Alexandre Jacques
FRENCH GUYANA
(20 samples)
Galion Forest
MARTINIQUE
(20 samples)
Chlorotypes
A
B
C
D
E
F
G
H
I
J
16°0'
4°0'
LATITUDE
0 500 1000 1500 Km
83°0'
LONGITUDE
52°0'

Fig. 4(b).